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14277

Mechanical Response of the Isolated Mammalian Heart to Anoxia.*

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The circulatory response to anoxia, in the intact animal, has been studied in a variety of ways and under a variety of circumstances.[1-7] All investigators are agreed that an increase in cardiac output occurs with moderate degrees of arterial desaturation. This response has been attributed[7] to a stimulating effect of anoxia directly upon the myocardium, though in no one set of experiments is it clear that all extra-cardiac factors have been rigorously excluded. Furthermore, experiments with the heart-lung and isolated heart preparations[8,9] under anoxia have not supported this view.

The present studies on the isolated heart were undertaken as part of a broader program to evaluate the factors in tolerance to altitude. When it became apparent that the myocardium, at least as examined in these experiments, was not a limiting factor, the studies, of necessity, were discontinued. However, a considerable body of data was accumulated bearing on the question alluded to at the outset, and it is essentially for this reason that a brief report of the work is here presented.

Method. Cats were used in all experi-

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[1] Harrison, T. R., Wilson, C. P., and Blalock, A., *J. Clin. Invest.*, 1925, **1**, 547.

[2] Sands, J., and DeGraff, A., *Am. J. Physiol.*, 1925, **74**, 416.

[3] Harrison, T. R., and Blalock, A., *Am. J. Physiol.*, 1927, **80**, 169.

[4] Harrison, T. R., Blalock, A., Pilcher, C., and Wilson, C. P., *Am. J. Physiol.*, 1927, **83**, 284.

[5] Gollwitzer-Meier, K., *Pfluger's Arch.*, 1928, **220**, 434.

[6] Strughold, H., *Am. J. Physiol.*, 1930, **94**, 641.

[7] Wiggers, C. J., *Ann. Int. Med.*, 1941, **14**, 1237.

[8] Gremels, H., and Starling, E. H., *J. Physiol.*, 1926, **61**, 297.

[9] Bogue, J. Y., Chang, I., and Gregory, R. A., *Quart. J. Exp. Physiol.*, 1937-38, **27**, 319.

ments, the heart being completely isolated according to a technic previously described,[10] except that the ventilating gas, or gas mixture, was passed directly from a tank through a moisture bottle and into the oxygenator, from which it escaped freely.

After cardiac output and cardiac volume had become stable, nitrogen was substituted for the oxygen used initially. In 7 of the 17 experiments in which complete flow readings were made, 5% CO_2 was used in both the oxygen and nitrogen. The resultant anoxia was progressive in character and provided a period of about 10 to 30 minutes, depending upon the preparation, during which the behavior of the heart over a wide range of oxygen tensions could be observed. After a measured and uniform increment in cardiac volume had occurred, oxygen, or oxygen + 5% CO_2 was readmitted.

The hearts in every case were fresh and in good condition. Except for minor fluctuations, filling and emptying pressures were maintained constant throughout.

In 8 additional experiments, 5 with and 3 without CO_2, measurements of the pH of arterial and venous bloods were made with the glass electrode at various stages of the procedure. Blood lactate values were followed in 3, and blood glucose and lactate values, in 4 other experiments. In 8 separate experiments, measurements of the oxygen content of the arterial blood were made with the manometric Van Slyke apparatus at various times. In all, over 40 experiments were carried out in 19 preparations.

Results. Cardiac volume. Behavior prior to the onset of dilatation varied with the presence or absence of CO_2 in the ventilating gas mixture. In the experiments without CO_2, with few exceptions, there was a slight to marked decrease in heart volume immediately following the onset of anoxia. When CO_2 was present, cardiac volume remained unchanged or increased slightly early in anoxia. Following this period, dilatation commenced, and, once established, progressed rapidly. At an arbitrarily selected volume increment, oxygen was readmitted. In those experiments carried out with CO_2 in the ven-

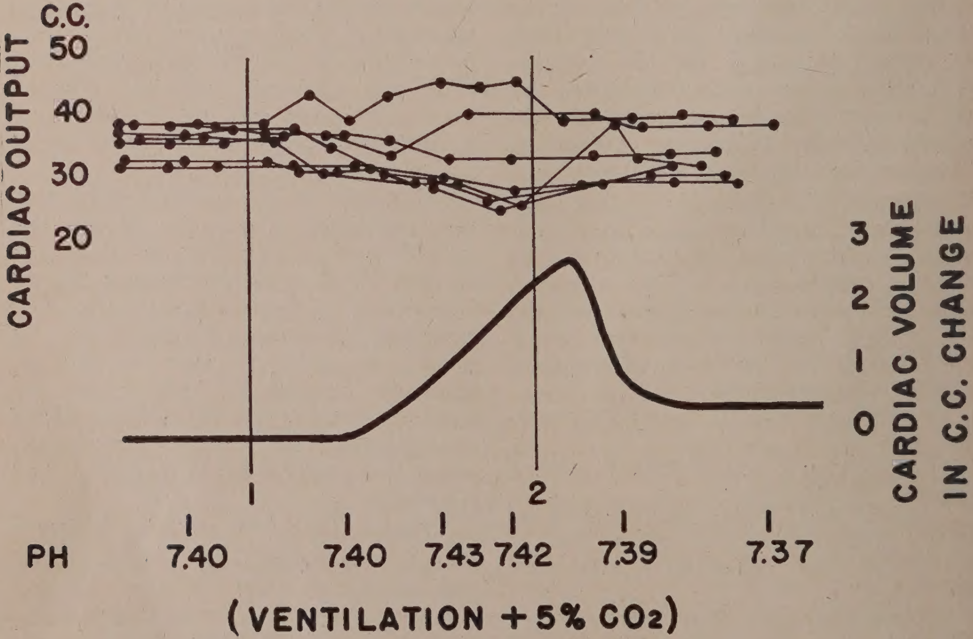

FIG. 1.

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[10] Lorber, V., *Am. Heart J.*, 1942, **23**, 37.

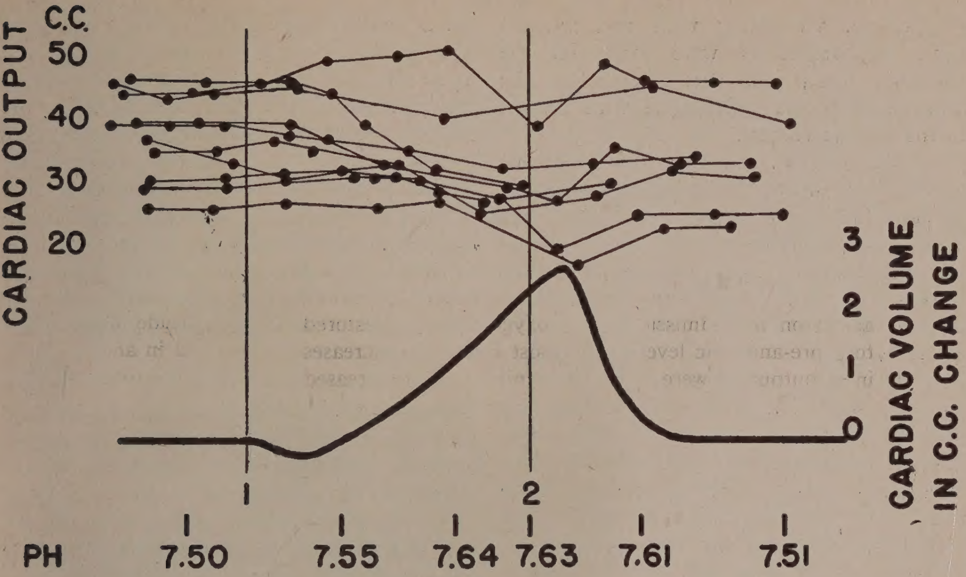

Fig. 2.

tilating mixture, recovery to the initial volume was not complete in most instances.

The time from the onset of anoxia to the readmission of oxygen varied widely from preparation to preparation (9 to 34 min.), but in any one preparation, the first two trials usually checked to within 1-2 minutes. With repeated trials, dilatation occurred more quickly. An effort was made to relate the dilatation times in some probable way to every physical and chemical factor of which measurements were made (including heart weight, coronary flow, external work, and blood volume, in addition to those already listed), but without success. The fact, however, that this response is a characteristic of the preparation, for whatever reason, permits each preparation to serve as its own control in experiments designed to test the influence of various agents on the response to anoxia.

The first detectable response to desaturation was an increase in the coronary flow. Dilatation did not begin, however, until oxygen tensions were very low. In 7 experiments in which measurements of the oxygen content of the arterial blood were made at the onset of dilatation, this occurred at about 50% (44 to 65) saturation, or at oxygen tensions of 10-15 mm Hg., under the conditions of the experiment (CO_2 absent from the ventilating gas). Subsequent experiments employing more gradual desaturation have been carried to even lower oxygen tensions before the appearance of dilatation. Though no measurements of oxygen were made, it was quite apparent that dilatation occurred much more readily in experiments in which CO_2 was present in the ventilating gas.

In the pH measurements a plausible explanation was found for the peculiar drop in cardiac volume seen early in preparations ventilated without CO_2. In these, increases of pH of as much as 0.2 unit were recorded in the course of desaturation, and appreciable differences were already in evidence when the volume dip appeared. In experiments with CO_2, on the other hand, elevations appeared later and only reached about one-third the magnitude noted above.

In Fig. 1 and 2, composite graphs of the results of experiments with and without CO_2, respectively, are presented. The heavy, peaked curves represent cardiac volume. The interval between ordinates 1 and 2 represents the period of anoxia. The pH values are from two experiments made on the same preparation, ventilating with and without CO_2. Measurements were made on the coronary blood and are entered on the abscissa at points appropriate with respect to the cardiac volume.

Cardiac Output. Of the 10 experiments without CO_2 in the ventilating gas, 5 showed an early increase in output of between 5 and 10%, one, a decrease of 10%, and 4, no significant change. Output tended to decline moderately with progressing desaturation, and on readmission of oxygen, was restored to pre-anoxic levels in most cases. Increases in output were accompanied by increased coronary flow, and decrements in emptying pressure of from 3 to 6 mm Hg due to the lowered peripheral resistance resulting from coronary dilatation. This factor alone, in the presence of constant filling pressure, could readily account for the increased output. However, it is apparent that if cardiac volume had been maintained constant in the early period, rather than permitting the usual fall, a further increase in output would have been recorded. In a subsequent experiment employing more gradual desaturation, a 32% increase in output was recorded early in the experiment, at constant cardiac volume and emptying pressure.

In the 7 experiments in which CO_2 was present in the ventilating gas, cardiac output was increased significantly (15%) early in anoxia in but one case, at which time the increment in coronary flow and decrement in emptying pressure were already quite appreciable. One late increase, when these factors were quite marked, was noted. Otherwise, the general course of the cardiac output was as described for the first group of experiments.

The results on output for all experiments are presented in Fig. 1 and 2. The interval between the ordinates 1 and 2 represents different time intervals in different experiments.

Discussion. When the completely isolated mammalian heart is subjected to anoxia, increased output that cannot be accounted for on a hemodynamic basis results only when CO_2 is absent from the ventilating gas, and is in all likelihood a function of the altered pH met with under these circumstances. As such, it must be regarded as a systematic artefact, rather than a physiological response of the cardiac muscle to anoxia. When 5% CO_2 is present in the ventilating gas, an increased output of purely cardiac origin is lacking. The elevations in the blood pH under these circumstances are similar in magnitude to those measured in the intact animal in anoxia.[3]

The caution with which results from experiments of this type must be considered in relation to problems involving the intact animal is emphasized by the recent experiments of Keys, Stapp, ond Violante.[11] These observers found altitude tolerance in normal young men increased by the presence of CO_2 in the inspired air. The isolated heart is affected adversely. However, the impression gained from the present experiments that cardiac muscle is hardly the limiting factor in tolerance to hypoxia, is well substantiated by Keys *et al.*, who found no evidence favoring a drop in cardiac efficiency at oxygen tensions at which syncope occurred.

Conclusions. 1. The depressant effects of anoxia on the completely isolated mammalian heart appeared at oxygen tensions well below those tolerated by the intact animal. The heart muscle, therefore, at least as examined in the present experiments, cannot be regarded as a limiting factor in tolerance of the intact animal for anoxia. 2. Increases in cardiac output (of purely cardiac origin) by the isolated heart under anoxia are seen only when CO_2 is absent from the ventilating gas. The effect appears to be due to the marked elevations in pH seen under these conditions, and, as such, is regarded as a systematic artefact rather than evidence of a direct stimulating effect of low oxygen tensions on the myocardium.

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[11] Keys, A., Stapp, J. P., and Violante, A., *Am. J. Physiol.*, 1943, **138**, 763.

14278

Effect of Follicular Hormone on Anaphylactic Shock.

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Numerous investigations have conclusively established that the anaphylactic response of a species can be modified by the endocrines. However, the literature of this field contains many conflicting statements. In a recent publication, in which he reviewed this literature, Farmer[1] pointed to the desirability of repeating some of these investigations.

The following experiments were undertaken to re-examine Schaefer's[2] report that large amounts of alpha-estradiol benzoate increased the susceptibility of sensitized guinea pigs to anaphylactic shock.*

Experimental. Seventy female guinea pigs, weighing an average of 280 g, were used. They were fed a diet consisting of oats, carrots, and greens. As there possibly exists a seasonal variation in the guinea pig's sensitivity to anaphylactic shock, control experiments and hormone experiments were done during the same season.

1. *Control experiments.* Thirty-three guinea pigs were used. They were sensitized by one intraperitoneal injection of 0.1 cc 1:10 diluted normal horse serum. Nineteen to 21 days after the sensitizing injection, and 18 to 24 hours after the last feeding, the animals received intravenously a shocking injection of 1:10 diluted normal horse serum; 4 animals were shocked with 0.5 cc, 6 with 0.6 cc, and 23 with 0.8 cc. Eleven of the 23 animals which received 0.8 cc, or 47.8%, died in shock. There were no deaths among the animals which received the smaller amounts of serum.

2. *Administration of alpha-estradiol benzoate.* In these experiments we slightly varied Schaefer's technic in that we used only one sensitizing injection as against Schaefer's 2 sensitizing injections, and in that we administered the hormone over a period of 11 to 13 days, whereas Schaefer gave the hormone over a period of 14 days. Thirty-seven guinea pigs were used. They were sensitized by one intraperitoneal injection of 0.1 cc 1:10 diluted normal horse serum. Beginning 8 days after this sensitizing injection, the animals were injected intramuscularly at 2 to 3 day intervals with 2,490 to 4,150 rat units of alpha-estradiol benzoate. Each animal received 4 to 5 injections, or a total of 12,450 to 16,600 units. Two days after the last hormone injection, 19 to 21 days after the sensitizing injection, and 18 to 24 hours after the last feeding, the guinea pigs were shocked by an intravenous injection of 1:10 diluted normal horse serum. Six animals received 0.5 cc, 6 animals 0.6 cc, and 25 animals 0.8 cc. Thirteen of the 25 animals which received 0.8 cc or 52%, died in shock. One of the 6 animals which received 0.5 cc died. There were no deaths among the animals which received 0.6 cc.

Conclusion and summary. A comparison of the control and alpha-estradiol experiments shows that administration of follicular hormone to serum-sensitized female guinea pigs did not enhance their anaphylactic response. This finding is in distinct contradiction to Schaefer's results.

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[1] Farmer, L., *Ann. Int. Med.*, 1942, **17**, 212.

[2] Schaefer, W., *Med. Klin.*, 1937, **33**, 1061.

* In Schaefer's experiments 28 out of 49, or 57%, of the sensitized guinea pigs which had received injections of follicular hormone died in anaphylactic shock, whereas 24 out of 73 control animals, or 33%, succumbed.

14279

A Note on the Distribution of β-Alanine

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Previous investigations of the distribution of β-alanine in various natural sources have been limited by the lack of sensitivity of the methods of analysis used. With the development of the diphtheria bacilli growth assay of β-alanine,[1] a method which is sufficiently sensitive to detect $5\ \gamma$ of the compound, it became possible to reinvestigate the problem more satisfactorily.

Kaplansky[2] used the method of Abderhalden and Fodor[3] for the detection of β-alanine to show that β-alanine was not present in muscle proteins. The method consisted of esterification of the protein hydrolysate and subsequent distillation of the esters. The characteristic odor of the acrylic ester formed by decomposition of the β-alanine ester was used for detection of β-alanine. By this method Kaplansky was able to find no β-alanine in extracted muscle, although some β-alanine not detected by this method was undoubtedly present in the hydrolysate since we have found that three extractions are not sufficient to remove all the β-alanine from muscle.

In the present study, a number of pure proteins, namely, zein, alpha wheat globulin, gliadin, canteloupe seed globulin, Bence-Jones protein, edestin (hemp seed), silk fibroin, egg albumin, and insulin were assayed and found to contain no β-alanine. Recently Pollack[4] has found by assaying with a yeast-growth method that silk fibroin, horse hemoglobin, egg albumin, gelatin, casein, and lactoglobulin were substantially free from β-alanine. It may be concluded that β-alanine is not a general constituent of proteins.†

Many attempts to isolate and estimate carnosine from various tissues may be found in the literature. Fewer attempts have been made to isolate anserine. By separating the tissues and assaying them individually for β-alanine, it would be possible to discover any considerable store of anserine or other β-alanine-containing compound. Table I shows the results of β-alanine assays of tissues obtained from a rat. Both the concentration and total amount of β-alanine are included. The liver and muscle tissues account for 90% of the total β-alanine and it is apparent that no large store of β-alanine compound exists in other tissues.

TABLE I.
Distribution of β-Alanine in the Rat.
(70 g female.)

	Mg per 100 g moist tissue	Mg in entire organ
Skeletal Muscle	25	4.1
Liver	26	0.9
Bones	0.8	0.04
Brain and Spinal Cord	2	0.03
Skin, Hair and Fat	0.6	0.08
Heart	<7	<0.02
Viscera (except liver)	2	0.27

Hydrolysis increases the activity of muscle extracts due to the liberation of free β-alanine which is more active than carnosine by the growth test. However, no such increase is observed upon hydrolysis of liver extracts. Smorodinzew[5] was unable to isolate carnosine from liver extracts. Furthermore, liver extracts were found to be more resistant to nitrous acid than solutions of β-alanine, carnosine, or muscle extracts, indicating that the amino group of β-alanine of the liver is protected. In view of these observations, the presence of much carnosine or anserine in liver is highly unlikely and the entire β-alanine content is probably in the form of pantothenic acid. The average values reported here are higher than the pantothenic acid assays reported by Strong, Feeney, and Earle,[6] and by Rohrman, Burget, and Williams,[7] but comparable to those of Williams and coworkers.[8,9]

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* Abbott Laboratories Research Fellow.

[1] Schenck, J. R., *J. Biol. Chem.*, in press.

[2] Kaplansky, S., *Z. physiol. Chem.*, 1926, **158**, 19.

[3] Abderhalden, E., and Fodor, A., *Z. physiol. Chem.*, 1913, **85**, 112.

[4] Pollack, M. A., *J. Am. Chem. Soc.*, 1943, **65**, 484.

† A preparation of phaseolin contained 0.07% β-alanine, but after some purification, the assay indicated only 0.02%; this amount was not further reduced by dialysis. The molecular weight of phaseolin is not known, but a molecular weight of 500,000 would be required by this low β-alanine content. It is more reasonable to consider the β-alanine as an impurity.

TABLE II.
β-Alanine Content of Hydrolyzed Tissue.
(Mg per 100 g of moist tissue.)

	Muscle			
Rat No.	Gastrocnemius	Pectoralis Minor	Liver	Kidney
105	—	—	28	5
118	—	—	26	—
130	—	—	19	5
162	100	6	< 2	—
196	105	—	15	<2
228	85	4	23	5
230	60	—	21	6
231	90	6	9	1
232	75	8	29	—
233	108	—	—	<1
235	—	—	—	5
239	95	4	—	<1
240	90	—	< 4	—
241	115	—	6	—
242	127	—	5	—

Cat No.	Gastrocnemius Muscle	Liver
III 41	—	25
III 50	650	—
IV 74	400	—
IV 95	350	—
IV 103	310*	—
V 52	—	9
V 75	500	6

* Autolyzed before hydrolysis.

Assays for β-alanine of tissues from the rat and the cat are presented in Table II. From this table it is apparent that there is considerable difference in the β-alanine content of different tissues, but that a specific tissue of one species has a fairly uniform β-alanine content in a number of individual animals. For example, in the rat, the gastrocnemius muscle contains about 100 mg % β-alanine, whereas the pectoralis minor contains about 5 mg %. The gastrocnemius muscle of the cat is about 4 times as rich in β-alanine as the corresponding tissue of the rat. Liver and kidney have a much lower β-alanine content than most muscle tissue.

Summary. The diphtheria bacilli growth test for β-alanine has been applied to a number of proteins and animal tissues. Skeletal muscle and liver were shown to be the richest animal sources of β-alanine, and β-alanine was shown to be absent from a number of purified proteins.

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[5] Smorodinzew, J., *Z. physiol. Chem.*, 1914, **92**, 214.

[6] Strong, F. M., Feeney, R. E., and Earle, A., *Ind. Eng. Chem.*, Anal. Ed., 1941, **13**, 566.

[7] Rohrman, E., Burget, G. E., and Williams, R. J., Proc. Soc. Exp. Biol. and Med., 1934, **32**, 473.

[8] Wright, L. D., McMahan, J. R., Cheldelin, V. H., Taylor, A., Snell, E. E., and Williams, R. J., *The University of Texas Publication*, No. 4137, 1941, 38.

[9] Mitchell, H. K., and Isbell, E. R., *The University of Texas Publication*, No. 4237, 1942, 37.

14280

Experimental Studies on Blood Sulfanilamide Levels Attained After Powdered Drug Administration by Inhalation.

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The possibilities of therapeutic effect and the dangers of overabsorption which might be produced by sulfanilamide administered by inhalation led to the following experiments. Harris, Sommer and Chapple[1] reported on the inhalation of sulfathiazole microcrystals (Smith, Kline and French) whereas our own experiments utilized ordinary dry sulfanilamide powder (Merck). In Harris, Sommer and Chapple's experiments white mice and guinea pigs were used as test animals. Blood levels of as high as 16.57 mg sulfathiazole/100 cc were observed in the mice which were kept for varying intervals of time up to 4 hours in a box containing the sulfathiazole "smoke." To test whether the absorption actually occurred in the lungs, tracheotomy and ligation of the esophagus was performed in 2 guinea pigs and the spray of microcrystals put into the trachea. Blood sulfathiazole levels attained were 3.46 and 1.93 mg/100 cc.

Harris *et al.*, also made therapeutic observations, finding that their inhalation procedure caused a great reduction in mortality due to inhalation of pneumococcus in mice suffering from influenza. Boissard and Fry[2] administered sulfanilamide powder by nasal insufflation to 26 children with a chronic mixed *C. diphtheriae* and hemolytic streptococcus infection of the nose. Good therapeutic results were obtained, but no blood sulfanilamide levels were reported nor was any attempt made to cause the sulfanilamide to go into the deeper portions of the respiratory tract.

Experiments. Bag method. Two dogs weighing 13.4 and 9.8 kg respectively were anesthetized with nembutal and a flannelette bag tied tightly around the snout of each of them. The flannelette was porous enough so that they could breathe through it. One end of the bag was attached to a blow bottle containing sulfanilamide powder through which was blown a current of air from an electric air pump. During the blowing of the air the blow bottle was continually shaken. The amount of sulfanilamide introduced into the animal was calculated approximately by estimating the amount blown out of the bottle and subtracting from this the amount caught in the interstices of the flannelette. This represents a maximum value and the true weight of drug inhaled is probably much less due to loss on the animal's hair and through the bag. The maximum

TABLE I.
Blood Sulfanilamide Levels Attained After Inhalation by the Bag Method in Two Dogs.

Dog	1			2		
Wt, kg	13.4			9.8		
Max. amt of Sulfanilamide, g	16			42		
Inhalation time	3:11-3:18; 3:21-3:33; 3:36-3:55 P.M., 12-11-42 Total = 38 min.			2:43-2:49; 2:51-2:58; 3:00-3:07; 3:18-3:22 P.M., 12-14-42. Total = 24 min.		
	Date	Time	Level	Date	Time	Level
Blood Sulfanilamide mg/100 cc	12-11	3:22 P.M.	1.0	12-14	3:30 P.M.	2.2
	,,	4:00 ,,	2.3	,,	4:50 ,,	2.4
	,,	11:50 ,,	1.3	,,	10:55 ,,	2.1
	12	10:15 A.M.	1.2	17	3:30 ,,	2.0
	21	11:00 ,,	neg.			

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[1] Harris, T. N., Sommer, H. E., and Chapple, C. C., *Am. J. Med. Sci.*, 1943, **205**, 1.

[2] Boissard, J. M., and Fry, R. M., *Lancet*, 1942, **1**, 610.

TABLE II.
Urine Sulfanilamide Levels Attained After Inhalation by the Bag Method in Dog 2.

Date	Time	Vol. urine specimen, cc	Urine sulfanilamide, mg/100 cc
12-14	5:30 P.M.	50	50
’’	11:05 ’’	30	250
15	5:00 ’’	90	60
17	3:30 ’’	10	trace

blood sulfanilamide levels attained were 2.3 and 2.4 mg/100 cc as shown in Table I. The blood levels showed a prompter rise than did the urinary drug levels as shown in Table II. These 2 dogs lived and were observed for some time following the inhalation and showed no ill effects.

Intratracheal method. In 3 additional dogs under nembutal anesthesia a catheter attached to the sulfanilamide powder blow bottle was inserted into the trachea through the larynx about as far as the bifurcation. Blood sulfanilamide levels attained by this method were considerably higher than by the other, the maximum levels reached being 33.2, 33.2, and 12.2 mg/100 cc in the 3 experiments as shown in Fig. 1. Brief protocols follow:

Experiment 3. Dog weight, 11.0 kg, less than 13 g sulfanilamide powder inhaled. Initial blood level of 12.2 mg/100 cc at 3:10 p.m. on 12-18-42, 8 minutes after cessation of 34 minutes of inhalation. Two days later the animal was very groggy, ate and drank little and coughed up considerable loose phlegm. At this time the high blood level shown in Fig. 1 of 20 mg/100 cc may have been due to swallowing coughed-up sputum. The early development of a high level of 12.2 mg almost immediately after the inhalation would, however, argue for a respiratory tract absorption. On December 21 the dog appeared normal except for a frequent

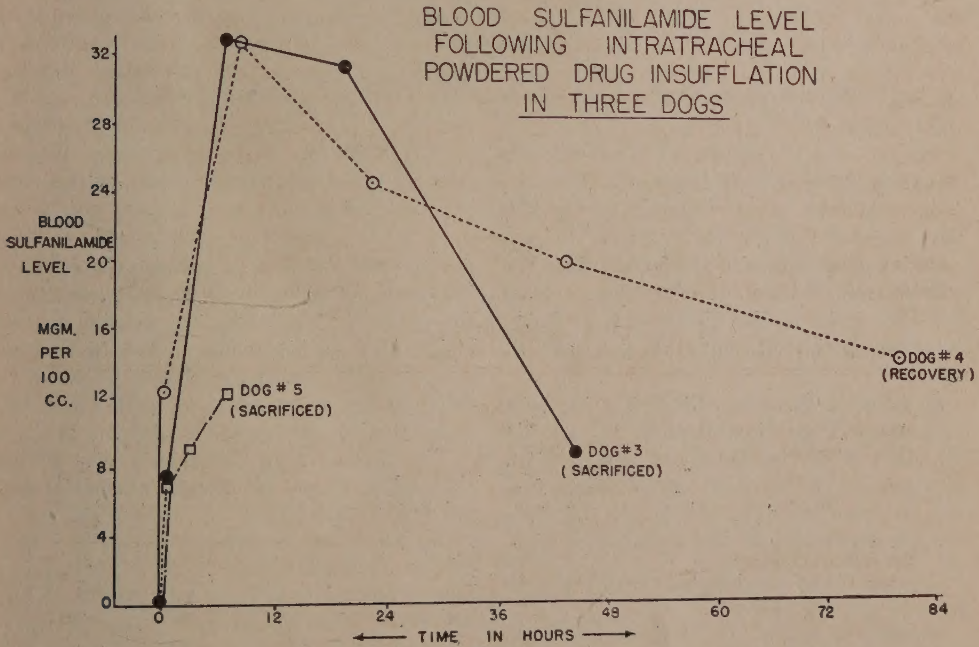

Fig. 1.

The rise in blood sulfanilamide concentration occurring after intratracheal powdered drug insufflation.

raspy, non-productive cough. It was killed with sodium cyanide and necropsy revealed nothing abnormal in any organs except the lungs, where long reddish yellow membranous strands of fibrinous material protruded from and partially plugged the main bronchi and some smaller bronchioles. Otherwise the lungs appeared normal and there was no evidence of sulfanilamide powder in the lungs or trachea.

Experiment 4. Dog weight, 18.4 kg, less than 17 g sulfanilamide powder inhaled. Initial blood level of 7.7 mg/100 cc at 4:40 p.m. on 12-18-42, 6 minutes after cessation of 50 minutes of inhalation. The animal had a harsh cough with practically no sputum during the first few days after inhalation. The dog was groggy on December 19 but was apparently normal the next day. The urine sulfanilamide level at noon the day after the inhalation was 1660 mg/100 cc. Large feathery crystals were seen in the test tube of urine. Recovery.

Experiment 5. Dog weight, 12.6 kg, less than 7 g sulfanilamide powder inhaled. Initial blood level of 7.1 mg/100 cc at 3:50 p.m. on 12-21-42, 3 minutes after cessation of 54 minutes of inhalation. The animal was killed with sodium cyanide 6 hours after the inhalation. Necropsy revealed nothing unusual in the kidneys, liver, heart, spleen, adrenals, or other organs except the trachea and bronchi. In the trachea and larger bronchi there were probably several grams of mucoid coated sulfanilamide while the smaller bronchioles showed only a small fleck of this material.

Summary and conclusions. The intratracheal insufflation of sulfanilamide powder in dogs will produce high blood levels of the drug (up to 33.2 mg/100 cc) associated with high urinary levels (up to 1660 mg/100 cc) and accompanied by general symptoms of depression and intoxication. A local accumulation of the powder persists during at least the first 6 hours and is followed by cough and signs of irritation of the lining of the trachea and bronchi which may last for several days.

14281

Attempts to Acclimate Poliomyelitis Virus to Tomato Plants.*

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It is now generally believed that the gastrointestinal tract of the human from the stomadeum to the hind gut is probably the main portal of entry of poliomyelitis virus.[1,2,3]

Because the gastrointestinal tract has been implicated, it has been thought by us that the virus might be found on and ingested with food. The common edible plants present no ostensible signs of infection, but it is possible that they may become infected without external or internal evidence of disease. We were not able, however, to demonstrate the presence of poliomyelitis virus in or on fruits, etc.,[4] although our failure to find it does not mean that it was absent. It was decided to see if poliomyelitis virus could be acclimated to some common plant.

The tomato was chosen for this work. Forty-eight tomato plants were planted in the university greenhouse and allowed to grow to an age where 4 separate shoots had appeared along the main stalk. They were then divided into sets of 6 and tested with one of the following materials: (a) "enteric" toxin, the filtrate obtained from coliform organisms growing in beef broth;[5] (b) poliomyelitis virus; (c) virus in "enteric" toxin and (d) sterile physiological saline. Two experiments were made with each test suspension, in the first of which a drop of material was placed on the fourth leaf from the tip of 6 plants and rubbed in with a smooth spatula; a second set of 6 plants were each injected with a drop of the same test substance just below the third internode. None of the 48 plants showed any reaction. Growth was neither disturbed nor stunted by the rubbing nor by the injections of any of the test suspensions.

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* Sponsored by the National Foundation for Infantile Paralysis, Inc.

[1] Toomey, John A., *J. Ped.*, 1941, **19**, 103.

[2] Toomey, John A., *Ann. Int. Med.*, 1935, **8**, 7.

[3] Toomey, John A., *J. Ped.*, 1936, **8**, 664.

[4] Toomey, John A., Takacs, W. S., and Tischer, Linda A., *J. Ped.*, 1943, **23**, 168.

We were not able to demonstrate any injury to the plant though it might be possible that virus could be present without producing any obvious disease in the host. Therefore, the plants which received virus were ground, the material filtered, and the fluid obtained was injected into one monkey (2 cc intracerebrally—I.C.), 3 Eastern cotton rats (0.06 cc I.C.—0.5 cc subcutaneously—S.Q.), 3 white rats (0.06 cc I.C.—0.5 cc S.Q.) and 3 mice (0.03 cc I.C.—0.5 cc S.Q.). No symptoms developed in any of the animals.

Thus, we have not been able to demonstrate that poliomyelitis virus can either live or multiply in the tomato plant.

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[5] Toomey, John A., Proc. Soc. Exp. Biol. and Med., 1934, **31**, 1015.

14282

Effect of Sodium Alkyl Sulfate on Peptic Activity of Gastric Contents in Man and *In vitro*.

Joseph B. Kirsner and Regis A. Wolff. (Introduced by Walter L. Palmer.)

From Frank Billings Medical Clinic and Department of Medicine, University of Chicago.

Recent studies by Shoch and Fogelson[1] and by us[2] have shown that sodium alkyl sulfate, a synthetic detergent of the anionic series, is capable of eliminating the peptic activity of gastric juice *in vitro*. This effect is produced without any significant deviation in pH. A similar inhibition of peptic activity by this detergent has been demonstrated *in vivo*[3] and has greatly increased the survival time of dogs with ulcers induced experimentally by the massive histamine injection technic described by Wangensteen and his coworkers.[4] The purpose of this communication is to present further data regarding the effect of sodium alkyl sulfate* on the peptic activity of the gastric contents in man and *in vitro*.

The gastric contents were partially aspirated in 3 adult male patients with duodenal ulcer every 2 hours from 8 a.m. to 8 p.m. The 8 a.m. sample served as a fasting control for each experiment. The peptic activity of the gastric contents was measured during the administration of 90 cc of an equal mixture of milk and cream alone at hourly intervals from 8:30 a.m. to 7:30 p.m. and together with varying amounts of sodium

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[1] Shoch, D., and Fogelson, S. J., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 304.

[2] Kirsner, J. B., and Wolff, R. A., to be published.

[3] Shoch, D., and Fogelson, S. J., *Quart. Bull. Northwest. U. Med. Sch.*, 1942.

[4] Walpole, S. H., Varco, R. L., Code, C. F., and Wangensteen, O. H., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 619; Varco, R. L., Code, C. F., Walpole, S. H., and Wangensteen, O. H., *Am. J. Physiol.*, 1941, **33**, 475.

* Supplied in generous amounts by Dr. S. J. Fogelson and by the Abbott Laboratories, North Chicago, Illinois. Chemical name, sodium lauryl sulfate; formula $NaC_{12}H_{25}SO_4$. Molecular weight, 288.3. The preparation contains 64.2% lauryl alcohol and 33.3% SO_4. It is a white crystalline solution of highest purity, soluble in water producing a neutral solution. Its principal property is that of reducing surface tension.

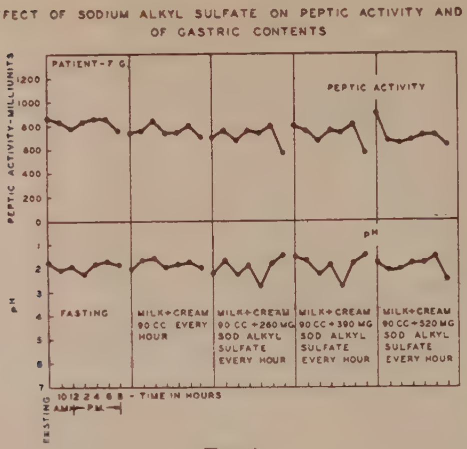

Fig. 1.

alkyl sulfate. The pH of the samples of gastric content was measured by the Beckman glass electrode. Peptic activity was determined in duplicate by the Beazell modification of the Anson-Mirsky method, adapted for the Evelyn photoelectric colorimeter.[5]

Sodium alkyl sulfate in doses of 130, 260, 390, 520 or 780 mg hourly did not produce a detectable lowering of peptic activity in man. The results of a typical series of experiments in one patient are shown in Fig. 1. Baker and her associates[6] had found previously that aqueous suspensions of lecithin, cephalin, and sphingomyelin prevented the inhibiting action of various synthetic anionic and cationic detergents on the respiration and glycolysis of gram-positive and gram-negative microörganisms. Information was sought therefore concerning the effect of sodium alkyl sulfate on peptic activity in 5 patients receiving a diet low in lipids. The results of a typical experiment are shown in Fig. 2. Peptic activity during the use of the special diet did not differ from that observed in previous control studies. The addition to the program of 780 mg of the detergent at hourly

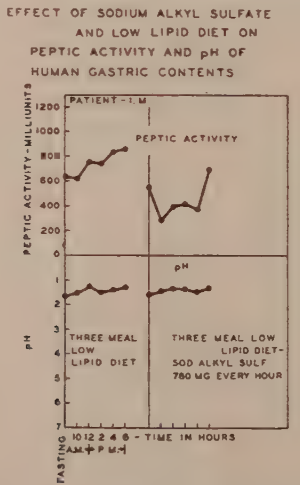

Fig. 2.

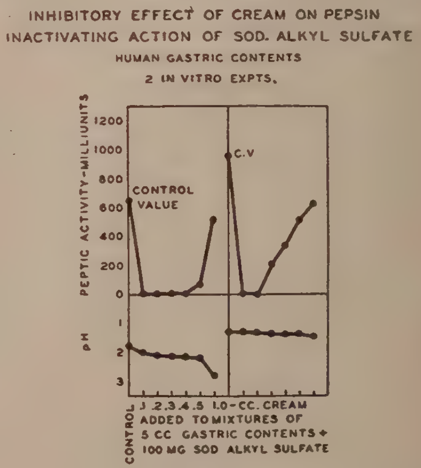

Fig. 3.

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[5] Warren, I. A., Front, J., and Kirsner, J. B., *Gastroenterology*, 1943, **1**, 102.

[6] Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **73**, 249; 1941, **74**, 621.

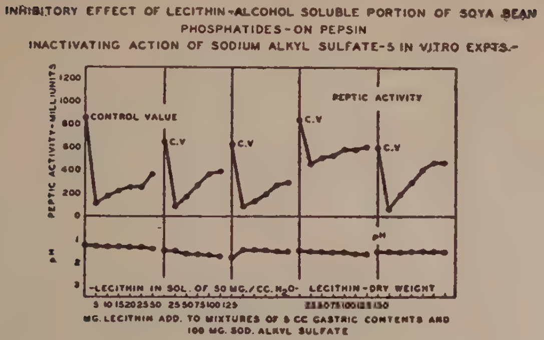

Fig. 4.

intervals resulted, however, in an appreciable decrease in peptic activity. This inhibitory effect was temporary and towards evening, at a time when gastric secretion increased, peptic activity rose to levels approximating the control values.

A series of *in vitro* experiments tended to confirm the above observation. Thus, the addition of small amounts of 20% cream to gastric juice before the addition of 100 mg of sodium alkyl sulfate resulted in a less effective inhibition of peptic activity by the detergent (Fig. 3). This effect was noticeable especially when 1.0 cc of cream was added. Similar results were obtained with ordinary butter. Five experiments in which increasing quantities of lecithin (the alcohol soluble portion of Soya bean phosphatides) were added to 5 cc amounts of gastric contents before the addition of 100 mg of sodium alkyl sulfate demonstrated that lecithin likewise retarded the effect of the detergent (Fig. 4). Lard and mineral oil, on the other hand, were practically ineffective. Olive oil exerted a slight effect.

Small quantities (0.1 cc) of pure triacetin, ethyl butyrate, ethyl caprylate, ethyl laurate, ethyl myristate, butyl stearate, and glycerin also retarded the pepsin-inactivating action of sodium alkyl sulfate; triacetin proved the most effective and glycerin the least effective (Table I). The degree of "protection" from the action of the detergent appeared to be greater with the fatty acid esters than with the other lipids tested. The pH of the gastric contents was not altered significantly in these experiments.

Conclusion. Sodium alkyl sulfate decreases the peptic activity of the gastric contents in man when administered in adequate doses in conjunction with a diet low in lipids. The pepsin-inactivating action of sodium alkyl sulfate is inhibited *in vitro* by cream, butter, lecithin, glycerin, and esters of fatty acids with carbon chains of varying length. Lard, mineral oil and olive oil are practically ineffective in this respect.

TABLE I.
Inhibition of Pepsin-Inactivating Action of Sodium Alkyl Sulfate by Esters of Fatty Acids and by Glycerin.

	Peptic activity (Milliunits per cc)	
Gastric juice alone	728	985
" " + 100 mg Sodium Alkyl Sulfate	0	0
" " " " " " " " + Triacetin	538	708
" " " " " " " " " Ethyl Butyrate	394	625
" " " " " " " " " " Caprylate	483	645
" " " " " " " " " " Laurate	466	686
" " " " " " " " " " Myristate	473	665
" " " " " " " " " Butyl Stearate	319	645
" " " " " " " " " Glycerin	149	559

0.1 cc quantities of fatty acid esters and glycerin added to 5 cc gastric juice before addition of 100 mg sodium alkyl sulfate.

14283

Erythropoietic Substance in the Serum of Anemic Animals.

Newton Krumdieck. (Introduced by D. P. Barr.)
With the technical assistance of Catherine Walcott.
From the Department of Medicine, Cornell University Medical College, and New York Hospital.

Hemorrhage or blood destruction from whatever cause is reflected in the bone marrow by erythrogenic hyperplasia. Chemical and mechanical factors are thought to be responsible. It is possible, however, that the stimulus to erythrogenic hyperplasia of the bone marrow is some substance in the blood serum of the anemic animal. Carnot and Deflandre[1] reported the presence of such a stimulus but Gordon and Dubin[2] on repeating their work were unable to demonstrate it. Yu Tin Tei[3] in an extensive series of experiments found a hemopoietic substance in the serum of anemic animals and described it as being alcohol, ether, chloroform, acetone soluble and heat stable at 100°C for 30 minutes. Its activity was reflected by increases in the red blood cells, hemoglobin and reticulocytes in the peripheral blood of recipient animals. The bone marrow was not studied in any of these observations.

The following experiments were designed to determine the effect of injections of serum of animals rendered acutely and chronically anemic by bleeding on the peripheral blood and bone marrow of normal recipient animals.

Healthy white male rabbits weighing between 2500 and 2800 g were used. Donor animals were bled from the marginal ear vein. Blood counts were done at approximately the

TABLE I.
Blood Counts of Rabbits Injected with Serum from Acutely Anemic Animals

	Rabbit No.	Hemoglobin in g/100 cc	Red blood cells in millions/mm³	Reticulocytes in %
Before injection	4	13.3	5.7	1.2
	5	13.7	6.0	1.8
	6	14.1	6.3	0.6
Day of ”	4	13.2	5.4	1.2
	5	13.5	5.9	1.1
	6	13.5	5.8	1.9
1 day after ”	4	13.7	5.2	1.6
	5	13.2	5.9	0.8
	6	13.6	6.6	1.2
2 ” ” ”	4	13.1	5.6	1.7
	5	13.0	5.1	4.2
	6	14.1	6.1	3.1
3 ” ” ”	4	13.3	5.7	6.3
	5	13.0	5.2	4.9
	6	13.0	5.9	6.8
4 ” ” ”	4	13.6	5.6	1.3
	5	13.5	5.2	2.1
	6	13.1	5.4	3.2
5 ” ” ”	4	13.2	5.5	2.9
	5	14.5	6.1	2.3
	6	13.7	5.8	3.2

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[1] Carnot and Deflandre, *Compt. Rend. de l'Acad.*, 1906, **143**, 384.

[2] Gordon and Dubin, *Am. J. Physiol.*, 1934, **107**, 704.

[3] Yu Tin Tei, *J. Chosen Med. Assn.*, 1938, **28**, 1, 32, 71, 129.

TABLE II.
Blood Counts of Rabbits Injected with Serum from Chronically Anemic Animals.

	Rabbit No.	Hemoglobin in g/100 cc	Red blood cells in millions/mm^3	Reticulocytes in %
Before injection	10	13.6	5.6	2.4
	11	13.4	5.2	3.1
	12	13.5	6.4	0.5
Day of ”	10	13.0	5.5	2.8
	11	12.6	4.7	3.7
	12	13.1	5.4	2.8
1 day after ”	10	13.5	5.9	4.2
	11	12.7	5.3	4.7
	12	13.1	5.6	5.2
2 ” ” ”	10	13.3	5.5	7.1
	11	13.1	6.0	6.3
	12	12.9	5.3	7.0
3 ” ” ”	10	12.9	5.9	10.5
	11	12.6	6.2	10.1
	12	13.2	5.6	8.9
4 ” ” ”	10	12.9	5.8	9.6
	11	12.9	5.5	9.0
	12	13.2	6.5	7.8
5 ” ” ”	10	12.8	5.9	8.6
	11	14.9	5.3	7.6
	12	13.2	6.8	7.0

TABLE III.
Blood Counts of Rabbits Injected with Serum from Normal Rabbit.

	Rabbit No.	Hemoglobin in g/100 cc	Red blood cells in millions/mm^3	Reticulocytes in %
Before injection	14	13.7	5.6	0.3
	15	14.0	5.1	2.1
	16	14.0	5.9	1.2
Day of ”	14	13.4	5.7	0.5
	15	13.9	5.2	1.1
	16	14.1	5.3	1.1
1 day after ”	14	13.7	5.2	1.2
	15	13.3	5.4	1.7
	16	13.2	5.7	0.4
2 ” ” ”	14	13.5	5.4	1.4
	15	13.6	5.3	0.7
	16	13.6	5.3	2.1
3 ” ” ”	14	14.0	5.4	2.3
	15	13.8	5.8	1.9
	16	13.6	5.3	2.1
4 ” ” ”	14	13.9	5.3	1.2
	15	13.3	5.0	0.4
	16	13.1	5.7	0.8
5 ” ” ”	14	13.3	5.7	2.5
	15	14.6	5.6	1.7
	16	13.8	5.8	0.4

TABLE IV.
Blood Counts of Rabbits Which Received No Serum.

	Rabbit No.	Hemoglobin in g/100 cc	Red blood cells in millions/mm^3	Reticulocytes in %
Control	17	14.1	5.4	0.1
	18	14.3	5.6	0.7
1 day	17	14.0	5.1	1.2
	18	13.8	5.3	1.0
2 "	17	14.1	5.7	0.2
	18	14.7	5.5	2.4
3 "	17	13.7	5.9	0.3
	18	13.8	5.1	1.9
4 "	17	13.9	5.7	1.3
	18	13.9	5.3	0.5
5 "	17	14.0	5.5	0.7
	18	14.2	5.8	1.3
6 "	17	14.6	5.3	1.0
	18	14.2	5.7	1.4

same time each day on venous blood obtained by pricking the marginal ear vein. Standard red blood cell pipettes and standardized Hellige hemoglobinometers were used. Reticulocytes were stained with brilliant cresyl blue using the usual coverslip technic. Touch preparations of bone marrow from the proximal ends of the femur, humerus and tibia were made at the time each animal was killed. These preparations were stained with Wright-Giemsa stain. Two hundred cells were counted in each preparation.

Experimental. I. Acutely anemic Donor Rabbits. Each of 3 rabbits had 30 cc of blood removed on the first and third days of the experiment. By this means the average original red blood cell count and hemoglobin value were reduced from 5.5 M/mm^3 and 13.0 g/100 cc to 3M and 7.9 g respectively. The reticulocytes increased from 2 to 12%. These animals were bled to death from the heart on the fourth day. The blood was allowed to clot and the serum was separated by centrifugation. The serum was pooled and 20 cc were injected intravenously into each of 3 normal rabbits which were killed on the fifth day after injection. The results of the blood counts are shown in Table I.

II. Chronically Anemic Donor Rabbits. Each of 3 rabbits had a total of 240 cc of blood removed from the ear vein by 8 semiweekly bleedings of increasing amounts. The average original red blood cell count and hemoglobin fell from 6. M/mm^3 and 13.2 g/100 cc to 3.8 M and 6.6 g. The reticulocytes rose from 3 to 25%. This degree of anemia had been maintained for 2 weeks before the animals were bled to death from the heart. The blood was allowed to clot and the serum was separated by centrifugation. Twenty cc of this pooled serum was injected intravenously into each of 3 healthy rabbits. The animals were killed on the fifth day after injection. The blood counts are shown in Table II.

Controls. I. Normal Donor Rabbit. Twenty cc of serum from a healthy rabbit were injected intravenously into each of 3 healthy rabbits which were killed on the fifth day after injection. The peripheral blood counts are in Table III.

II. Normal Rabbits. Two healthy rabbits received no serum but blood counts were done each day. These are listed in Table IV. The bone marrow cell counts for all groups are detailed in Table V.

The bone marrow of the acutely anemic donor rabbits showed a slight increase in the proportion of erythrogenic cells. The bone marrow of rabbits injected with serum from these donor animals showed a normal proportion of erythrogenic and myelogenic cells.

TABLE V.
Average Bone Marrow Cell Counts of All Groups.

Experimental	Rabbit No.	Erythrogenic cells %	Myelogenic cells %
I. Acutely anemic donor rabbits	1	59	41
	2	64	36
	3	63	37
Recipients of serum from acutely anemic rabbits	4	58	42
	5	65	35
	6	47	53
II. Chronically anemic donor rabbits	7	88	12
	8	85	15
	9	78	22
Recipients of serum from chronically anemic rabbits	10	66	34
	11	67	33
	12	66	34
Control	13	55	45
1. Recipients of normal serum	14	58	42
	15	55	45
	16	56	44
II. Normal	17	51	49
	18	52	48

The bone marrow of the chronically anemic rabbits was hyperplastic as demonstrated by the large number (78-88%) of erythrogenic cells in it. Recipients of serum from these donor animals had bone marrows in which the number of erythrogenic cells was increased compared to normal.

Discussion. In neither the recipient nor the control animals was there a significant change in the red blood cell count or hemoglobin. In each of the experimental recipient groups there was an increase in reticulocytes which reached a peak on the third day after the injection of serum from the anemic animals. The reticulocyte count ranged between 4.9 and 10.5%. That this is significant is indicated by the fact that the normal reticulocyte count in rabbits varies usually between 0.5 and 2.0% and rarely is greater than 3.0%. In contrast to the experimental recipients the control groups showed no increase above the normal range of reticulocytes. The erythrogenic to myelogenic cell ratio in the bone marrow of the group which received serum from the acutely anemic animals showed no conspicuous deviation from that of normal animals. The erythrogenic to myelogenic cell ratio of the group which received serum from the chronically anemic animals was slightly increased above the normal.

Conclusions. These experiments suggest the presence of an erythropoietic substance in the serum of animals which had been rendered anemic by bleeding.

14284

Serological Properties of Products Synthesized from Sucrose by Enzymes from Different Strains of Leuconostoc Bacteria

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Previous reports[1,2] established the fact that sterile enzyme solutions obtained from *Leuconostoc mesenteroides* have the capacity to synthesize a serologically reactive polysaccharide from sucrose. These studies also showed, for one strain of leuconostoc, that the polysaccharide which was formed in the absence of cells agreed in major chemical respects and in serological properties with the dextran elaborated in cultures of the living bacteria. The purpose of the present paper is to present more complete evidence on the serological agreement between the products formed by the enzyme solutions and those formed in living culture. The new experiments were made with representatives of 2 serologically different types of leuconostoc bacteria.[3,4] Strains of one type are characterized by the fact that their sucrose broth culture fluids cross-react in high dilution with types 2, 12 and 20 antipneumococcus serums; whereas the culture fluids of strains of the second type react in high dilution with types 2 and 20 antipneumococcus serums, but react only in low dilution with type 12 serum.

Sterile enzyme solutions were prepared[2] from sucrose broth cultures of 6 strains of *Leuconostoc mesenteroides* representing the 2 described serological types. Material synthesized from sucrose by the enzymes was obtained for serological study as follows: 1 part of each enzyme solution was added aseptically to 3 parts of a sterile solution of 5% sucrose in 0.1 M acetate buffer (pH 5.6); the enzyme-sucrose mixtures were then incubated for 5 days at 23°C, together with appropriate controls. The sterility of all the mixtures was proved by rigorous microscopic and cultural tests.

The incubated enzyme-substrate mixtures, together with supernatant fluids obtained from sucrose broth cultures of the 6 different strains, were tested in a series of dilutions against types 2, 12 and 20 antipneumococcus rabbit serums. The serum-antigen mixtures were incubated for 1½ hours at 37°C and were then observed for occurrence of precipitation. The type-specificity of the reactions was controlled by tests against types 1 and 8 pneumococcus antiserums.

The results of the serological tests of the incubated enzyme-sucrose mixtures and of the sucrose broth culture fluids are summarized in Table I. The results of the tests of the incubated control mixtures (enzyme plus glucose, enzyme plus buffer, heated-enzyme plus sucrose) are omitted because none of them showed any increase of reactive material over the small amount originally contained in the enzyme solutions. The reactions obtained with the crude enzyme-sucrose mixtures and culture fluids can be accepted as the reactions of dextran polysaccharides, since dextran is the only leuconostoc product known to cross-react with types 2, 12 and 20 antipneumococcus serums.

It is evident (Table I) that the products formed from sucrose by the enzymes from the 6 different strains showed the same likenesses and differences in serological properties as occurred among the products formed in living cultures of the strains from which the enzymes had been derived. That is, the

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* Aid was received by a grant from the Ruth B. Ettinger Fund.

[1] Hehre, E. J., *Science*, 1941, **93**, 237.

[2] Hehre, E. J., and Sugg, J. Y., *J. Exp. Med.*, 1942, **75**, 339.

[3] Sugg, J. Y., and Hehre, E. J., *J. Immunol.*, 1942, **43**, 119.

[4] Neill, J. M., Sugg, J. Y., Hehre, E. J., and Jaffe, E., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 339.

TABLE I.
Serological Comparison of Products Formed from Sucrose by Sterile Enzyme Solutions with Products Formed in Living Cultures.

Strain of leuconostoc	Reactivity *vs.* types 2, 20 and 12 antipneumococcus serums			
	Enzyme-sucrose mixtures incubated 5 days at 23°C		Supernatant fluids of sucrose broth cultures	
	2 and 20	12	2 and 20	12
B	++++	+	++++	+
M	+++	0	++++	+
O	++++	+	++++	+
A	+++	+++	+++	+++
C	++++	++++	++++	++++
K	+++	+++	++++	++++

++++, precipitation with 1:5000 or 1:10000 dilution of the antigens.
+++, ” ” 1:500 or 1:1000 ” ” ” ”
+, ” ” 1:10 or 1:20 ” ” ” ”
0, no precipitation with 1:10 ” ” ” ”

enzyme-sucrose mixtures of strains *B, M* and *O,* like the culture fluids of those strains, had only slight capacity to react with type 12 in comparison to their capacity to react with types 2 and 20 antipneumococcus serum; whereas the enzyme-sucrose mixtures of strains *A, C* and *K,* like the culture fluids of those particular strains, had as high capacity to react with the type 12 as with the types 2 and 20 antiserums.

It is important to note that this comparison, based on the *ratio* of reactivities with 3 different antiserums, is an unusually critical sort of test which is capable of detecting a minor serological difference even among products which appear alike if tested against a single antiserum. Consequently, the agreement shown in Table I in respect to the *ratio* of reactivities against the 3 types of antipneumococcus serum is an especially significant index of close serological likeness between the materials formed from sucrose by sterile enzymes and the materials formed in living cultures of the strain of leuconostoc bacteria from which the enzyme had been derived.

14285 P

Protection of Normal Rats Against Death from Water Intoxication with Adrenal Cortical Substances.*

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It is well known that, in general, resistance to stress is low in adrenalectomized animals and that protection can be afforded by appropriate replacement therapy. Similarly adrenalectomized animals are susceptible to the intoxicating effects of excess water, and studies have been made on the effects of various steroid substances in repairing this deficiency.[1] Attempts to increase the re-

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† John Simon Guggenheim Memorial Fellow.

‡ The author wishes to express his indebtedness to Professor W. W. Swingle, in whose laboratory this work was conducted, for the laboratory facilities and cortical extracts used here; and to Dr. H. W. Hays, Ciba Pharmaceutical Products, Inc., for supplying desoxycorticosterone (Percorten).

[1] Eversole, W. J., Gaunt, R., and Kendall, E. C., *Am. J. Physiol.,* 1942, **135**, 378.

TABLE I.
Average Figures Concerning the Effect of Adrenal Cortical Substances on Animals Given Lethal Doses of Water.

Exp. No.	No. animals	Treatment	Water dose, cc	Times water given	% water excreted at following hours:				Rectal Temp. °F at following hours:				Chloride excreted (as mg NaCl)	No. convulsive	No. died
					1st	4th	7th	9th	1st	4th	7th	9th			
I.	3	9 cc 0.8% NaCl	9.7	11-15	15	56	61			90	86		97.6	3	3
	3	9 cc Cort. Ext.	10.0	15	11	59	69			96	96		98.6	1	0
II.	6	9 cc 0.8% NaCl	10.0	11-19	7	43	55	49	99	91	87	85	113.4	6	6
	6	9 cc Cort. Ext.	10.0	19	8	60	68	73	99	95	93	92	146.8	1	0
III.	6	1.7 cc P-nut oil	9.3	11-18	8	32	40	51	100	92	87	85	67.9	6	6
	6	12 mg DCA	9.2	18*	23	50	56	58	100	94	89	88	97.6	4	1

* One died after the 15th dose of water; the rest received 18 doses.

sistance of intact animals to stress by the use of cortical substances have been generally (*e.g.*),[2,3] although not in all instances (*e.g.*),[4,5] unsuccessful. The experiments reported here show that a striking protection can be afforded normal animals against the intoxicating effects of water by either a whole extract of the adrenal cortex or desoxycorticosterone acetate (DCA).

Methods. Routine procedures were similar to those previously used.[1] Male rats, fasted 18 hours and weighing approximately 200 g were used. Distilled water (3 cc per 100 sq cm of body surface) was given by stomach tube at half-hour intervals until all of the controls in each of the 3 experimental groups were dead. In the treated animals, 1 cc of cortical extract was given per hour, beginning just before the first dose of water. Controls received 0.8% NaCl similarly. In the DCA-treated series, 5 mg was given subcutaneously 18 hours before the experiment, 5 mg intramuscularly one-half hour before water was started, and 2 mg intraperitoneally during the experiment. Controls received peanut oil in amounts equal to that used as a solvent for the DCA. Experiments on both treated and control animals in each group were run simultaneously. The cortical extract used was a concentrated preparation in which 1 cc represented 200 g of whole gland.

Results. The results are shown in Table I. Two experiments were conducted with cortical extract. The controls were killed by 11 to 15 doses of water in one case, and 11 to 19 in the other. All treated animals received the highest dose of water given to any one of the controls, and all survived. The treated animals also surpassed their controls by the other criteria observed, *i.e.*, maintenance of body temperature, rate of diuresis, and freedom from the typical convulsions of water intoxication. It is well known that saline itself is helpful in preventing water intoxication,[6] so that the comparison here was between two beneficial agents.

DCA was also beneficial, but did not maintain the animals as well, relative to their controls, as did the cortical extract. One DCA-treated animal died before all the controls were dead, and 3 others were greatly weakened and convulsive. The latter 3, however, survived and appeared normal the following day. Since standards by which to establish comparable dosage and methods of administration of the extract and DCA as used here are lacking, the inferiority of the latter in this experiment should not be emphasized. It is, however, consistent with the better results obtained by extract or 17-

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[2] Huizenga, K. A., Broffman, B. L., and Wiggers, C. J., *J. Pharm. and Exp. Ther.*, 1943, **78**, 139.

[3] Swingle, W. W., Overman, R. R., Remington, J. W., Kleinberg, W., and Eversole, W. J., *Am. J. Physiol.*, in press.

[4] Selye, H., Dosne, C., Bassett, L., and Whittaker, J., *Canad. Med. Assn. J.*, 1940, **43**, 1.

[5] Katz, L. N., Killian, S. T., Asher, R., and Perlow, S., *Am. J. Physiol.*, 1942, **137**, 79.

[6] Rowntree, L. G., *J. Pharm. and Exp. Ther.*, 1926, **29**, 135.

hydroxy-11-dehydrocorticosterone as compared to DCA in adrenalectomized animals given large doses of water.[1]

The chloride excretion figures are not to be interpreted as measures of renal activity, since the urine samples were contaminated with diarrheal fluid, particularly in the controls. There is a loss of considerable fluid through the intestine in terminal stages apparently due to an eventual failure of water absorption from the intestine.[7] The chloride figures are quoted to show, however, that the protective action of the cortical substances is not through a prevention of the loss of this ion from the body.

While a more detailed study of the action of cortical steroids in the response to excess water will be reported elsewhere, it is perhaps noteworthy that most types of stress in which cortical substances have been given intact animals without consistent success have been shock-producing stimuli, associated with marked hypotension. Water intoxication is a different type of phenomenon in that blood pressure is well maintained until near the time of death.[6,7,8]

Summary. Large doses of adrenal cortical extract or desoxycorticosterone acetate will protect normal rats from doses of water which otherwise produce lethal water intoxication.

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[7] Gaunt, R., unpublished observations.

[8] Swingle, W. W., Parkins, W. M., Taylor, A. R., and Hays, H. W., *Am. J. Physiol.*, 1937, **119**, 557.

14286 P

Hair Loss in Male Dogs Fed Stilbestrol.

R. M. MULLIGAN. (Introduced by A. R. Buchanan.)

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Failure of regeneration and loss of hair have been reported[1] in dogs given estradiol benzoate. No account has been found of the same phenomenon in dogs which received synthetic estrogens.

In a recent experiment* 4 male dogs received *by mouth diethylstilbestrol,*† 5 mg tablets, over a period of several months. These animals and 2 other male dogs (serving as controls) were also injected subfascially with methylcholanthrene, the changes resulting from which will be described elsewhere. All of the animals were mongrels about 1½ to 3 years old at the start of the experiment. The weights ranged between 15 and 27 lb. and did not vary significantly.

The first dog received 330 mg of stilbestrol in 20 doses of 5 mg, 19 of 10 mg and 2 of 20 mg in a period of 129 days, at the end of which the hair was missing from the right flank.

The second dog received 1790 mg of stilbestrol in 20 doses of 5 mg and 169 of 10 mg in a period of 292 days, at the end of which the hair was generally thinned, was largely missing from the abdomen and penis, and had failed to regenerate on the forelegs at areas clipped for vein puncture.

The third dog received 1850 mg of stilbestrol in 185 doses of 10 mg in a period of 280 days, at the end of which the hair was missing from the thighs, buttocks, perineum, abdomen, face, and the ventral surface of the tail, thorax, and neck.

The fourth dog received 2985 mg of stilbestrol in 100 doses of 10 mg, 6 of 15 mg, 6 of 20 mg, and 71 of 25 mg in a period of 291 days, at the end of which the hair was absent from the base of the tail, the lumbar regions, the scapular regions, the abdomen,

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[1] Gardner, W. U., and DeVita, J., *Yale J. Biol. and Med.*, 1940, **13**, 213.

* Aided by a grant of the Council on Research from the fund of the Graduate School of the University of Colorado.

† Furnished through the courtesy of Dr. D. C. Hines of Eli Lilly and Company.

the penis, the circumocular areas, and the ventral surface of the jaw, and had failed to regenerate on the forelegs at areas clipped for vein puncture.

The fifth and sixth dogs used as controls showed no loss of hair and normal regeneration of hair on the forelegs at areas clipped for vein puncture. The 3 experimental animals receiving the largest doses of stilbestrol revealed the maximum loss of hair in the middle of the winter of 1942-43, when the period of observation was terminated and when the 2 control dogs and several other dogs in the runs employed in other, non-hormonal experiments had a uniformly thick coat of hair.

In view of the loss of hair in male dogs following the peroral administration of stilbestrol over several months, a similar phenomenon might be anticipated in humans, particularly in the light of a recent clinical note[2] describing the loss of hair from the head of a young woman who had been given stilbestrol for several months.

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[2] *J. A. M. A.*, 1943, **121**, 224.

14287

Acid Phosphatase Content of Nuclei of Chicken Erythrocytes.

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The recent discovery of 6 important enzymes in nuclei isolated from rat liver cells[1] has made it seem desirable to extend enzyme investigations to an entirely different type of nucleus. In this work we have investigated the acid phosphatase content of isolated chicken erythrocyte nuclei which now can be obtained in relatively undamaged condition.[2,3] The nuclei were prepared by the method of Lan and Dounce[3] from washed erythrocytes prepared from fresh defibrinated chicken blood obtained at the slaughter house. A photograph of these nuclei is shown in Fig. 1. Acid phosphatase was chosen for the investigation because it is relatively easy to determine and already has been reported in whole erythrocytes.[4,5] Moreover, it is one of the enzymes found in isolated liver cell nuclei.[1]

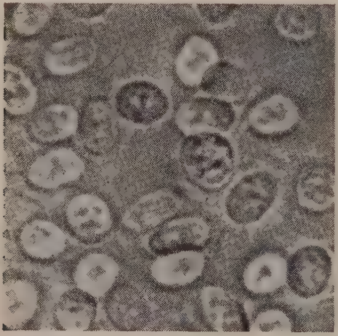

FIG. 1.

Isolated nuclei of chicken erythrocytes, oil immersion, unstained.

The method employed for the determination of the enzyme was a slight modification of Gutman and Gutman's adaptation[6] of the original alkaline phosphatase method of King and Armstrong[7] to the determination of acid phosphatase. In this method, buffered disodium phenyl phosphate is used for substrate. The buffered substrate is made by mixing equal parts of 0.22% disodium phenyl phosphate and 0.4 molar acetate buffer adjusted to give a pH of 5.0 when diluted one to two. The substrate and buffer are mixed immediately before use.

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[1] Dounce, A. L., *J. Biol. Chem.*, 1943, **147**, 685.

[2] Laskowski, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 354.

[3] Dounce, A. L., and Lan, T. H., *Science*, 1943, **97**, 584.

[4] Roche, J., and Bullinger, E., *Enzymologia*, 1939, **7**, 278.

[5] Roche, J., *Biochem. J.*, 1931, **25**, 1724.

[6] Gutman, E. B., and Gutman, A. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 513.

[7] King, E. J., and Armstrong, A. R., *Canad. Med. Assn. J.*, 1934, **31**, 376.

In carrying out the determinations, 0.5 cc portions of a suspension containing from 5 to 10 mg of nuclei on a dry weight basis were added to 10 cc portions of buffered substrate in test tubes, and the tubes then were placed in a thermostat bath held at 25°C and were agitated from time to time. The reaction was stopped by the addition of 5.0 cc of the diluted phenol reagent, and the test tubes were placed in the icebox for 10 to 15 minutes to allow the precipitated material to agglutinate. The precipitate was removed by filtration, the filter paper was washed once with distilled water, and then 3.5 cc of 15% sodium carbonate solution were added to the clear filtrate. The test tubes were heated in boiling water for 5 minutes to develop the color, then cooled in tap water, and finally the contents were filtered. The filter paper was washed once with distilled water, and the filtrate was diluted to 25 cc. The amount of color developed was read in a photoelectric colorimeter. Blank determinations were run at the same time on samples which had not been incubated with substrate. The readings obtained from the blank runs were subtracted from corresponding readings obtained from the incubated samples, in order to obtain values to be used in calculating the amount of phenol liberated by the enzyme from the substrate. Dilute phenol in 0.2 M acetate buffer of pH 5.0 was used in preparing standards of known concentration.*

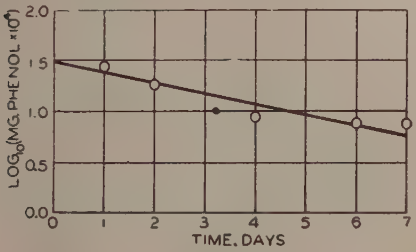

FIG. 2.

Rate of decay of acid phosphatase of chicken erythrocyte nuclei. Ordinate = log$_{10}$ (10^4 times mg phenol liberated) per mg nuclei, dry weight, by incubation with substrate for 1 hr. Abscissa = age of preparation of nuclei in days, stored at approximately 3°C.

Although the concentration of acid phosphatase found in the isolated erythrocyte nuclei was rather low (about 15 to 20 % of the concentration reported for the nuclei of normal rat liver cells),[1] its value was readily measurable. The average acid phosphatase concentration of fresh preparations of nuclei is sufficient to cause the liberation of 0.0025 to 0.0030 mg of phenol from the substrate per mg of nuclei (dry weight) per hour at 25°C and pH 5.0.

The activity of the acid phosphatase of chicken erythrocyte nuclei diminishes with time, even when the isolated nuclei are kept in the icebox at approximately 3°C, so that fresh preparations must be used if maximum activity is to be obtained. The rate of decay of enzyme activity when the nuclei are kept at about 3°C is shown in Fig. 2. The activity of the enzyme is immediately abolished if a suspension of the nuclei is boiled.

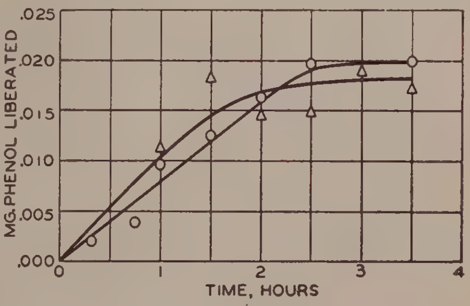

FIG. 3.

Action of acid phosphatase of chicken erythrocyte nuclei. ○ = 6.5 mg nuclei dry weight. △ = 5.2 mg nuclei, dry weight. Ordinate = mg phenol liberated from substrate by action of the enzyme. Abscissa = time of incubation of nuclei with substrate.

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* The above procedure is the same as that which was used but not described in detail for the determination of acid phosphatase in nuclei isolated from normal rat liver,[1] except that 0.5 cc of a suspension of erythrocyte nuclei was substituted for 0.1 cc of a suspension of liver cell nuclei.

During incubation of the nuclei with the substrate, the nuclei remain in unbroken condition, since it has been found impossible so far to break them up without causing agglutination into a single mass of material. This fact may mean that the measured enzyme activity of the nuclei is somewhat lower than the true activity, since diffusion of substrate into the nuclei and diffusion of reaction products out of the nuclei may be limiting rate factors.

In the course of the determination of the acid phosphatase in isolated chicken erythrocyte nuclei, a curious phenomenon was observed, which is illustrated in the curves shown in Fig. 3. These curves show amounts of phenol liberated from the substrate by given weights of nuclei, plotted against time. The curves go up almost linearly and then flatten out rapidly at the end of a period of about 2 hours. Further incubation of the nuclei with the substrate results only in the slow uncatalyzed decomposition of the substrate. We have attempted to ascertain the cause of this abrupt loss of enzyme activity, and have finally concluded that it may be the effect of the substrate in inactivating the enzyme, although this has not been proved directly. The effect is not caused by the addition of as much as 0.04 mg of phenol to the nuclei before incubation with the substrate. Addition of 0.047 mg of phosphate buffer before addition of the substrate also caused no apparent inhibition of the enzyme. Furthermore, nuclei kept at 25°C for 2 hours and 15 minutes in the presence of buffer alone at pH 5.0 (the pH employed in the determination of the enzyme) showed no decrease in enzyme activity. This length of time is greater than the length of time necessary to reach the flat portion of the substrate decomposition-time curve.

It should be kept in mind that the nuclei of chicken erythrocytes prepared by the method employed in this work are surrounded by an extremely tenuous stroma, generally invisible except with the oil immersion objective after staining. Such a stroma also was noticed surrounding nuclei prepared by the method of Laskowski.[2] However, it is improbable that this stroma can contribute more than a minor amount to the observed enzyme activity of the preparations, since it does not appear to constitute a very appreciable fraction of the mass of the nuclear material.

We have found that the activity of washed whole chicken erythrocytes is about the same as that of the isolated nuclei.

Summary. 1. The enzyme acid-phosphatase has been determined in nuclei isolated from washed chicken erythrocytes. The activity of fresh preparations determined by the use of disodium phenyl phosphate as substrate corresponds to the liberation of about 0.0025 to 0.0030 mg of phenol from the substrate per mg of tissue per hour at 25°C. Approximately the same concentration of enzyme has been found in the washed whole erythrocytes. 2. After incubation of the nuclei with the substrate for a period of about 2 hours, the activity of the acid phosphatase quickly drops to a negligible value.

14288

Clinical Studies on a New Pepsin Inhibitor.*

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Dogs in which gastric ulcers were produced by daily histamine injections were kept alive longer than usual when the peptic activity of the gastric juice was inhibited by sodium lauryl sulfate which did not alter the pH.[1] In *in vitro* experiments sodium lauryl sulfate completely inhibited peptic activity without alteration of the pH.[2] We have therefore studied the effect of sodium lauryl sulfate on the peptic activity of gastric juice of patients suffering from peptic (gastric, duodenal or stomal) ulceration.

Procedure. Peptic ulcer patients, proven by x-ray, were used for this study. A Rehfuss tube was introduced into the stomach of the fasting patient and the gastric contents aspirated. With the tube *in situ*, the patient received 2 crackers and one glass (200 cc) of water. Samples of gastric juice were aspirated every 15 minutes for 2 hours. Determinations of the pH (by the Beckman Electrometer) and peptic activity (by a modification[3] of the Anson-Mirsky Hemoglobin Method)[4,5] were made on each specimen. On succeeding days the same procedure was repeated but immediately after the test meal the patient received either (a) 100 or (b) 200 mg of sodium lauryl sulfate, or (c) 2 g of calcium carbonate, or (d) 8 cc of an aluminum hydroxide preparation. Some patients were given 100 or 200 mg of sodium lauryl sulfate immediately following the fourth aspiration. Each patient was tested for 5 successive days.

TABLE I.

Average of the pH and Peptic Activity as Obtained in 24 Peptic Ulcer Patients Following (a) Ewald Meal and (b) Ewald Meal Plus Calcium Carbonate, Aluminum Hydroxide, or Sodium Lauryl Sulfate (aspirations at 15-minute intervals). Representative values of samples taken at 15 minutes, 1 hour and 2 hours shown.

	Fasting stomach		Aspirations		
			15 min	1 hr	2 hr
pH	2.8	Ewald test meal	2.6	1.5	1.7
Peptic activity*	1,620		1,150	1,570	1,760
pH	2.9	Ewald plus calcium carbonate (2 g)	5.4	2.2	1.7
Peptic activ.	1,450		710	1,450	1,720
pH	3.2	Ewald plus aluminum hydroxide (8 cc)	5.1	2.5	1.8
Peptic activ.	1,400		650	1,230	1,710
pH	2.6	Ewald plus sodium lauryl sulfate (100 mg)	2.7	2.0	1.7
Peptic activ.	1,310		1,040	1,520	2,090
pH	2.8	Ewald plus sodium lauryl sulfate (200 mg)	3.1	1.7	1.8
Peptic activ.	1,310		970	1,570	1,750

*Peptic activity measured in milliunits.

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* Aided by a grant from the Nutrition Research Laboratories, Chicago.

[1] Shoch, David, and Fogelson, S. J., *Quart. Bull. Northwestern Univ. Med. School*, 1942, **16**, 2.

[2] Shoch, David, and Fogelson, S. J., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 304.

[3] Beazell, J. M., Schmidt, C. R., Ivy, A. C., and Monaghon, J. G., *Am. J. Dig. Dis.*, 1938, **5**, 661.

[4] Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1932, **16**, 59.

[5] Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol*,. 1938, **22**, 79.

TABLE II.
Average of the pH and Peptic Activity as Obtained in 12 Peptic Ulcer Patients Following Ewald Test Meal and After Ewald Test Meal Plus Sodium Lauryl Sulfate at Hourly Intervals (aspirations at half-hour intervals).

	Fasting stomach		1 hr		2 hr
pH	3.7	Ewald test meal	1.7		1.7
Peptic activity	1,250		1,010		1,460
pH	3.6	Ewald plus sodium lauryl sulfate (100 mg)	1.7	Sodium lauryl sulfate (100 mg)	1.3
Peptic activ.	1,210		1,200		1,520
pH	3.9	Ewald plus sodium lauryl sulfate (100 mg) (duplicate test)	2.0	”	1.8
Peptic activ.	1,070		1,200		1,430
pH	3.7	Ewald plus sodium lauryl sulfate (200 mg)	3.0	Sodium lauryl sulfate (200 mg)	1.8
Peptic activ.	1,040		1,190		1,580
pH	3.6	Ewald plus sodium lauryl sulfate (200 mg) (duplicate test)	1.9	”	1.9
Peptic activ.	1,480		1,270		2,050

* Peptic activity measured in milliunits.

Results. The results agreed fairly closely between determinations on these patients on various test substances; regardless of the initial pH and peptic activity, the response to the test substance was similar. Thus nearly all patients responded markedly to calcium carbonate and to the aluminum hydroxide preparation and comparatively little to sodium lauryl sulfate. Slight variation in response to sodium lauryl sulfate was noted when it was given on successive days. The entire results are shown in Tables I and II, each of which represents the average of the pH and peptic activity of the cases studied.

Discussion. Following the simple test meal there was a rather slow drop of the pH in all cases. The peptic activity decreased slightly at first but then rose so that after 2 hours it was higher than at the beginning of the test period. Calcium carbonate and the aluminum hydroxide preparation caused the greatest rise of pH and the greatest decrease in peptic activity for the longest time (45 to 60 minutes) but after 2 hours the values for either were similar to those obtained with the test meal (Table I). Neither pH nor peptic activity were significantly affected by the administration of 100 mg sodium lauryl sulfate (the average peptic activity, rather, being highest during this test day). After 200 mg of sodium lauryl sulfate a slight rise in pH and a decrease in peptic activity were noted.

The administration of a second dose, 100 or 200 mg, of sodium lauryl sulfate after 1 hour did not influence pH or peptic activity (Table II). In all cases, peptic activity was associated with alteration (drop) of pH. Failure of sodium lauryl sulfate to cause inhibition of peptic activity in peptic ulcer patients, on the usual medical regimen was also noted by Kirsner and Wolf.[6] The latter, however, noted inhibition of peptic activity if the diet was free of phospholipids.

Summary. Calcium carbonate and an aluminum hydroxide preparation caused inhibition of peptic activity and a rise in pH of the gastric juice of peptic ulcer patients. One hundred mg of sodium lauryl sulfate caused no significant changes, whereas 200 mg caused a slight rise in pH and decrease in peptic activity. In the presence of unchanged pH no pepsin inactivation by sodium lauryl sulfate occurred.

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[6] Kirsner, Joseph B., and Wolf, Regis, personel communication.

14289 P

Return of Function of Sweat Glands After Cutting or Crushing Sympathetic Nerves.*

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Although considerable information is available concerning the regeneration of somatic nerves and the return of functions controlled by them, little is known concerning the regeneration of peripheral autonomic nerves.[1] The galvanic skin reflex and measurements of changes in cutaneous resistance and temperature when used as indices give information on whether regeneration is taking place but these methods do not give information quantitatively, on the extent of the return of peripheral autonomic activity which has taken place.[2]

In the present experiments measurements of the rate of sweating in the pads of the paws of cats have been made before and after the sympathetic nerves to them have been cut, to provide such data. The method is that of Neumann, Cohn and Burch,[3] adapted to the needs of these experiments. The hind paw is enclosed in an air-tight chamber through which is passed a continuous stream of dry oxygen. The dry gas takes up water lost by the paw and carries it into a tared metal coil immersed in a freezing mixture of alcohol and solid CO_2. The water suspended in the gas is deposited in the coil. After a suitable collection period (15 minutes) a new coil is substituted so that the collection of the water is continuous. The water-laden coil is dried and weighed. The difference in the weights before and after removal of water represents the water output for the period of collection. The method is accurate to 3%.

The water output from the pads of the normal right and left hind paws (area of paw approximately 30 sq cm, area of pad approximately 6 sq cm) was measured in 25 animals. When animals are at rest and the body temperature is normal (37 to 38.5°C) the water output reaches a basal level of 7 to 10 mg/15 min. This amount represents water lost by evaporation in the absence of active sweating from the pad. The same basal rate of water loss occurs from areas of skin devoid of sweat glands. The animals were then subjected to radiant heat in a cabinet, from which the paws were excluded, for a period of 45 minutes; the rectal temperature reached 40 to 41°C. The rate of water output increased 4 to 6 times (30 to 50 mg/15 min). The increase above the original or initial rate results from active sweating and is used as a quantitative measure of the activity of the autonomic fibers and will be referred to as the response. It is unnecessary in this connection to regard other functions for water loss in the bodies of cats.

Following unilateral denervation either by section and suture or by crushing of the sciatic and saphenous nerves the water output remained unchanged in the denervated leg and was without response to the influence of heating while the opposite normal leg responded as before. Eighteen animals were operated on, 10 by section and suture at the level of the popliteal space; 8 by crushing.

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* This is the 13th paper reporting the results of studies of the small blood vessels and related subjects.

† Fellow of the University of Cincinnati, working on a grant from the Josiah Macy, Jr., Foundation.

The Bureau of Medicine and Surgery does not necessarily undertake to endorse views or opinions which are expressed in this paper.

[1] Young, J. Z., *Physiol. Rev.*, 1942, **22**, 318.

[2] Richter, C. P., and Whelan, F., *J. Neurophysiol.*, 1943, **6**, 191.

[3] Neumann, C., Cohn, A. E., and Burch, G. E., *Am. J. Physiol.*, 1941, **132**, 748.

Measurements of the loss of water were made periodically from the normal and denervated sides. In the series denervated by section and suture, 15 to 30 weeks elapsed before the first sign of response was observed. The amount of the response thereafter increased gradually. After an average of 41 weeks the rate of water output reached an average of 42% of that put out by the control side.

In the animals in which denervation had been accomplished by crushing, the return of the response was quicker and more complete. It commenced an average of 15 weeks after operation and in the nineteenth week had risen to 69% of that of the control side. In 4 of the 8 animals, complete return of function was attained; the others showed rapid increases in function with each succeeding week.

Comments. The amount of the return of the response of the sweat glands on heating the body of the cats measures, we believe, the amount of recovery of the sympathetic fibers. After the crushing operation, almost twice the function returned in less than half the time than when section and suture were employed. The possible role of atrophy of the sweat glands as a contributing factor in the result cannot be fully measured, but the complete return of function in 4 animals suggests that the glands retain their ability to respond as soon as innervation is restored.

Crushing the nerve does less permanent harm, obviously, than cutting it. This result resembles what has been found in somatic nerves and probably on the same basis—less interruption to the pathways over which regeneration takes place.[4] The present method of estimating the amount of return of function in peripheral sympathetic nerves may be useful in forecasting the rate and amount of recovery after injuries to peripheral nerves in human beings. The method may, of course, have value in deciding which procedures are best in facilitating the regeneration of injured nerves[5] and may have application in the clinical study of central nervous lesions and of drugs affecting the secretion of sweat.

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[4] Gutmann, E., and Sanders, F. K., *J. Physiol.*, 1943, **101**, 489.

[5] Craig, W. M., *U. S. Naval Medical Bulletin*, 1943, **41**, 613.

14290

Utilization of Biotin and Biotin Methyl Ester by *Lactobacillus casei.*

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Kögl noted that biotin and its methyl ester are equally active for the growth of yeast (*Saccharomyces cerevisiae,* strain M).[1] Subsequently, it has been shown that biotin methyl ester can be utilized by a number of bacteria which require biotin for growth: *S. aureus,*[2] *B. radicicola,*[3] *Cl. butylicum,*[4] *Streptobacterium plantarum*[5] and a hemolytic streptococcus (C203S);[6] also by various

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[1] Kögl, F., and Pons, L., *Z. physiol. Chem.*, 1941, **269**, 61.

[2] Kögl, F., and Van Wagtendonk, W. J., *Rec. trav. chem.*, 1938, **57**, 747.

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[5] Möller, E. F., *Z. physiol. Chem.*, 1939, **260**, 246.

[6] Hottle, G. A., Lampen, J. O., and Pappenheimer, A. M., *J. Biol. Chem.*, 1941, **137**, 457.

TABLE I.

Availability of Biotin and Biotin Methyl Ester for *L. casei*.

2.0 mg of biotin methyl ester in 20 ml of methanol was diluted with water to .0005 γ in 5 ml. Biotin was similarly diluted from a stock solution of 48.88 γ per ml of 20% ethanol. 5 ml of each solution was added to an equal volume of double-strength medium.

Substance	Treatment	Assay medium of Landy and Dicken	 Shull *et al.*
		ml 0.1 N acid formed	
Biotin methyl ester (No. 1)	Autoclaved	10.4	4.0
” ” ” ”	Seitz filtered	8.8	3.2
” ” ” (No. 2)	Autoclaved	9.1	3.6
” ” ” ”	Seitz filtered	10.0	4.1
Biotin	Autoclaved	8.8	4.5
Distilled water (5 ml)	Seitz filtered	1.5	0.9
” ” ”	Autoclaved	1.6	0.4

fungi,[7,8,9] pea embryos,[10] the rat[11] and the chick.[12] It is presumed that these biological systems can convert the methyl ester to biotin-free acid. The ester is readily hydrolyzed by mild treatment with acid[1] or alkali.[1,13]

In contrast to the equal activities of biotin and biotin methyl ester for yeast, the latter is only about 10% as potent as biotin for *S. plantarum*.[5] The rat, also, may not utilize the ester as efficiently as the free acid.[14]

Recently it was reported that *Lactobacillus casei* cannot utilize biotin methyl ester and must be supplied with the free acid for growth.[15] However, data obtained by us repeatedly under carefully controlled conditions, indicate that *L. casei* can utilize the methyl ester, although growth is somewhat slower than with the free acid. The reason for the difference in results reported from the two laboratories is not apparent.

Methods. The general procedure and medium described by Landy and Dicken[16] in their assay method for biotin was used. Folic acid was kindly supplied by Dr. R. J. Williams.

Unless otherwise indicated, the experiments were made with a strain of *L. casei* originally obtained from Dr. Williams and used for the past two years for the assay of riboflavin and pantothenic acid. Two other strains were also used, one employed by Shull *et al.*[15] and obtained from those investigators and the other from the American Type Culture Collection (No. 7469).

Crystalline biotin and biotin methyl ester were used. The latter was added to the medium within 2 to 3 hours after solution in methyl alcohol to minimize possible hydrolysis and a fresh solution of the crystalline material was prepared for each experiment. A stock solution of biotin in 20% ethanol was prepared and used throughout. The ester was sterilized either by autoclaving it in the medium or by filtration through a Seitz E-K pad or a sintered glass filter, after which it was added aseptically to the sterilized medium. Biotin was autoclaved in the medium.

Experiments. Table I shows that essentially maximum acid formation by *L. casei* occurs with two different preparations of the methyl ester of biotin as well as with biotin. There is no significant difference in the re-

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[7] Kögl, F., and Fries, N., *Z. physiol. Chem.*, 1937, **249**, 93.

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[10] Kögl, F., and Haagen-Smit, A. J., *Z. physiol. Chem.*, 1936, **243**, 209.

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[15] Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, 1942, **142**, 913.

[16] Landy, M., and Dicken, D. M., *J. Lab. Clin. Med.*, 1942, **27**, 1086.

TABLE II.
Rate of Growth and Acid Formation of *L. casei* with Biotin Methyl Ester and Biotin.*

	1 day		2 days		3 days	
	% transmissible light†	ml 0.1 N acid	% transmissible light	ml 0.1 N acid	% transmissible light	ml 0.1 N acid
Biotin methyl ester	85	1.0	47	6.2	39	9.6
	89	1.1	44	6.1	41	9.8
	85	1.0	48	6.1	39	9.2
Biotin	56	3.1	37	9.4	32	10.3
	56	3.2	36	9.3	33	10.1
	55	3.3	37	9.2	33	10.1
No biotin	99	0.4	94	0.5	94	0.7

* Both compounds (.001 γ/10 ml of medium) were autoclaved in the medium.
† Determined with an Evelyn photometer.

sults with autoclaved and Seitz filtered solutions of the methyl ester thus eliminating the possibility that growth of the lactobacillus is due to thermal hydrolysis of the ester to biotin. For some unknown reason, the amount of acid formed in the medium of Shull *et al.* was only about half that to be expected. However, the trend of the results follows that noted in Landy's medium.

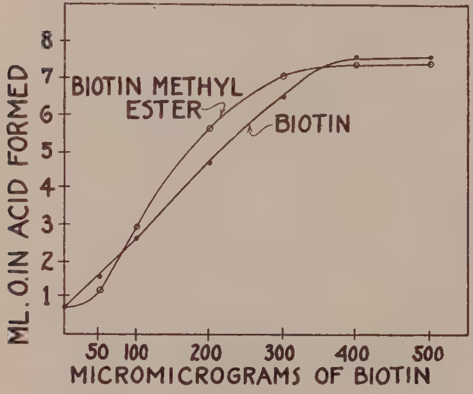

FIG. 1.
Formation of acid by *L. casei* with biotin and biotin methyl ester.

The initial pH of the medium (pH 5.8 to 7.6) does not affect the ability of *L. casei* to develop with biotin methyl ester.

The Wisconsin and A.T.C.C. strains of *L. casei* also developed abundantly in Landy's medium containing biotin methyl ester and formed as much lactic acid (7 to 8 ml, N/10) as with biotin.

Fig. 1 shows that, quantitatively, biotin and its ester* are equally available to the lactobacillus as judged by titratable acidity of the cultures after 3 days incubation. However, when the course of growth and fermentation is followed periodically, it is noted that the cultures develop more slowly with the ester than with the free acid (Table II). Very little growth is evident with the ester after 1 day, and 3 days are required for maximum acid formation as compared to 2 days with biotin. This lag may be a measure of the time required for conversion of the ester to the physiologically active free acid. The sequence of initial slight growth followed by abundant growth in biotin methyl ester medium is not due to gradual hydrolysis of the ester by the medium itself, since the same sequence occurs if the medium is stored at 37° for as long as five days prior to inoculation.

Nine serial subcultures of *L. casei* in biotin methyl ester medium did not increase its rate of growth.

Discussion. Esters of vitamins required by *L. casei* for normal development are generally inactive or nearly so for this organism: ethyl monacetyl pantothenate and ethyl pantothenate are only slightly active;[17] riboflavin tetraacetate is inactive;[18] and pyridoxine triacetate is inactive although the diacetate is almost as active as pyridoxine itself.[19] The slow initial growth with biotin methyl ester indicates that this ester also, is used with some difficulty.

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* Sterilized by filtration through a sintered glass filter since Seitz pads may adsorb appreciable quantities of the growth factor especially from dilute solutions.

Since the retardation in growth and acid formation of *L. casei* with biotin methyl ester as compared to biotin, may still be evident after 3 days incubation, it is preferable to use biotin as a standard or vitamin supplement in assay methods involving this organism.

Summary. In common with other organisms which require biotin for growth, *L. casei* can utilize the methyl ester of biotin. However growth and fermentation are slower than with free biotin.

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[17] Unna, K., and Mushett, C. W., *Am. J. Physiol.*, 1942, **135**, 267.

[18] Snell, E. E., and Strong, F. M., *Enzymologia*, 1939, **6**, 186.

[19] Bohonos, N., Hutchings, B. L., and Peterson, W. H., *J. Bact.*, 1942, **44**, 479.

14291

Excretion of Penicillin in Bile.*

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Florey demonstrated in animals that bile contained penicillin after the intravenous exhibition of the antibacterial substance.[1] The present study was undertaken in order to determine whether it is excreted similarly in the bile in man.

A solution containing 1,000 Oxford units of the sodium salt of penicillin[†] per cubic centimeter in 0.85% sodium chloride was injected intravenously in all patients studied. In 5 patients a double lumened tube was passed under fluoroscopic guidance so that gastric and duodenal contents (bile) could be collected separately by means of constant suction by the method of Agren and Lagerloff.[2] Specimens were obtained every 15 minutes and placed immediately in the refrigerator. Samples of blood were withdrawn several times and the serum separated by centrifugalization.

The concentration of penicillin in the serum was determined by a method described previously.[3] The pH of the gastric juice and bile was obtained just prior to the determination of the concentration of penicillin. The penicillin in the bile and gastric juice was assayed by the same method as was used with the serum except that in these samples *Staphylococcus aureus* was used as the organism since hemolytic streptococci did not grow satisfactorily. The smallest amount that could be detected was about 0.078 Oxford units per cubic centimeter of bile or gastric juice.

Results. Fig. 1 shows the concentration of penicillin in the blood and in the bile in 3 of the 5 patients studied after an intravenous injection of 20,000 Oxford units. The concentration of penicillin in the serum one minute after the injection varied from 0.625 to 2.5 Oxford units per cubic centimeter. There then followed a rather rapid decrease so that 120 minutes later only small amounts of the bactericidal substance could be de-

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* Supported by a grant from the Johnson Research Foundation, New Brunswick, New Jersey.

[1] Abraham, E. P., Florey, H. W., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., and Jennings, M. A., *Lancet*, 1941, **2**, 177.

† Penicillin used in this study was supplied through the courtesy of Dr. George A. Harrop, Squibb Institute for Medical Research, New Brunswick, New Jersey.

[2] Agren, G., and Lagerloff, H., *Acta Med. Scandinav.*, 1936, **90**, 1.

[3] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

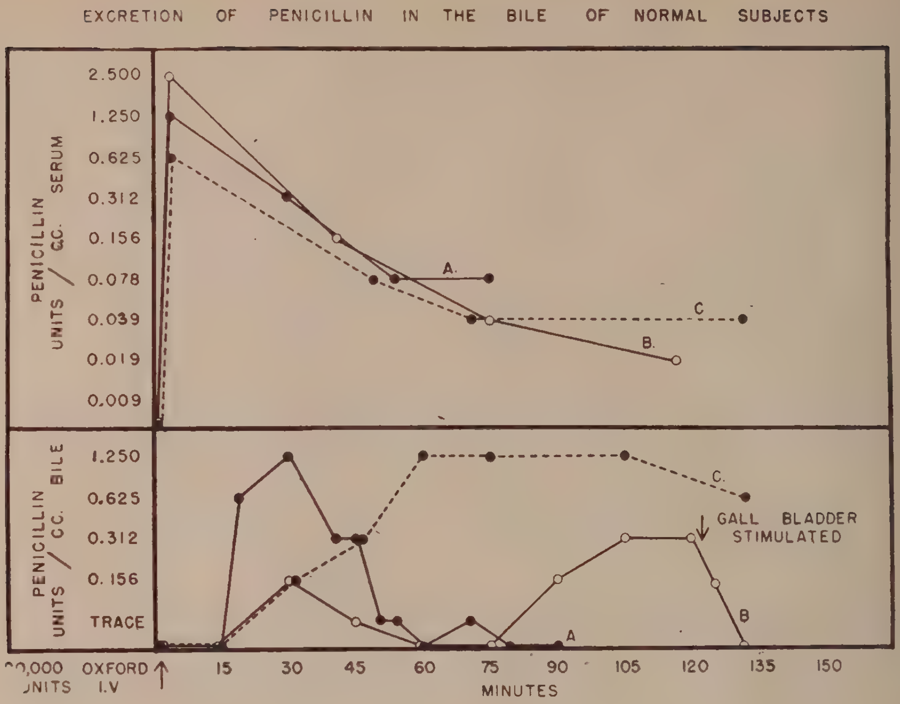

FIG. 1.

tected in the serum.

Gastric juice aspirated every 15 minutes failed to show any penicillin as determined by the tests described, but the bile did contain considerable amounts of the substance. It is to be noted that the first specimen obtained 15 minutes after the administration of penicillin exhibited no antibacterial activity. Specimens obtained at 20 and 30 minutes contained significant amounts of penicillin.

Of interest is the variation in the curves of the concentration of penicillin in the bile in these three subjects. Subject A reached a maximum level at 30 minutes, and then fell rather rapidly. In contrast is curve B which reached its highest level of 0.312 Oxford unit per cubic centimeter 105 minutes after the injection. At 120 minutes, following the administration of a small amount of magnesium sulfate through the tube, the concentration of penicillin in the bile diminished rapidly.

In subject A the pH of the bile at 30 minutes was 7.2, whereas the remainder of the specimens of this bile showed a pH varying from 3 to 6. In the 2 subjects not shown in Fig. 1 the bile contained no penicillin. This may be explained by the observation that the duodenal contents of one subject were white suggesting that no bile was obtained, while in the second subject the test for penicillin was unsatisfactory because of contamination of the cultures.

In view of the fact that the 5 patients had intact, functioning gall bladders, 20,000 Oxford units were given intravenously to a patient recovering from a cholecystectomy, the bile being collected by means of a T-tube in the common bile duct. Fig. 2 shows the concentrations of penicillin in the serum and bile of this subject. The first sample of bile obtained 15 minutes after the injection contained 1.25 Oxford units per cubic centimeter, whereas the serum at 5 minutes contained only 0.625 Oxford unit. The concentration of penicillin in the bile fell gradually

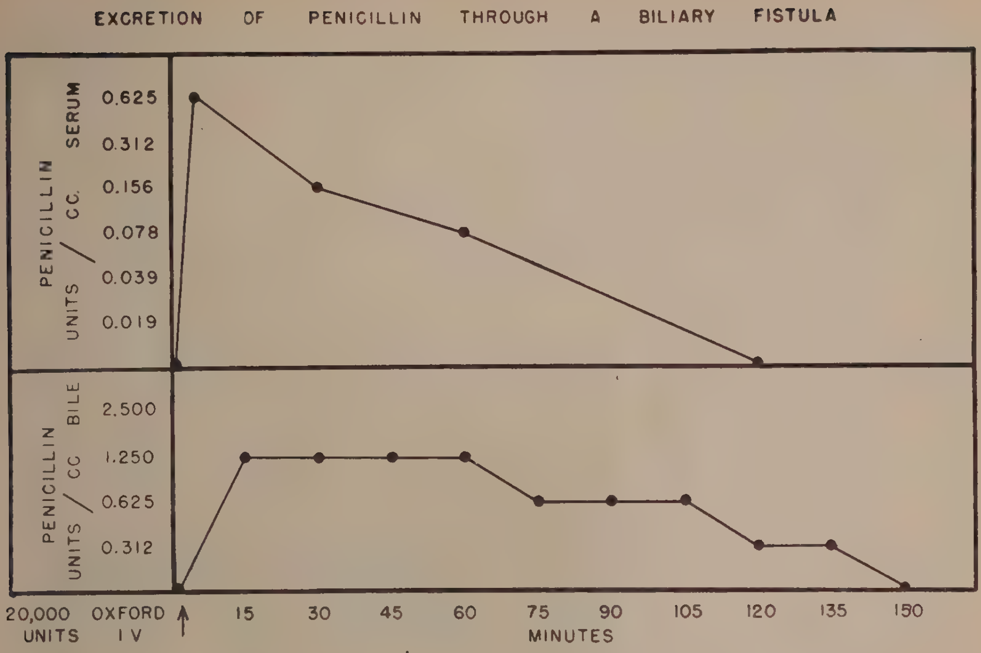

Fig. 2.

and this is in contrast to the results obtained in those subjects with normal gall bladders. The total amount of penicillin excreted in the bile in this subject was 64 Oxford units.

Discussion. It has been demonstrated that saliva, bile and succus entericus do not inactivate penicillin, whereas gastric juice obtained from normal subjects rapidly destroys its antibacterial properties.[4] The acid in the gastric juice is apparently responsible for the inactivating effect.[4] In the present study the administration of penicillin to normal subjects resulted in the excretion of the substance in the bile, but not in the gastric juice. In subject A, it was noted that somewhat irregular levels of penicillin were obtained in the bile and this was thought to be due to the inadvertent passage of gastric juice into the duodenum thus lowering the pH and destroying the penicillin. Further, the irregular curves of the concentration of penicillin in the bile might be accounted for by the periodic emptying of the gall bladder, resulting in dilution of the common duct bile. That this may play a role is suggested by the rapid lowering of the concentration of penicillin in subject B after the stimulation of the gall bladder.

When penicillin was given to a subject without a gall bladder, it appeared in the first specimen of bile obtained at 15 minutes and thereafter the concentration diminished gradually. It is of some interest that the concentration of penicillin was greater in the bile than in the serum in several instances, suggesting that the material was concentrated by the liver.

It is now well recognized that considerable amounts of penicillin are destroyed in the body but the explanation for this loss is not quite clear. The fact that a subject with complete diversion of the bile excreted only 64 Oxford units makes it seem unlikely that loss of penicillin by this route can account for an appreciable amount of that destroyed.

Since penicillin appears in the bile in concentrations which have been shown to be effective against gram-positive organisms,[5] it would seem that this substance should prove useful in the treatment of certain infections of the biliary system.

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[4] Rammelkamp, C. H., and Helm, J. D., Jr., submitted for publication.

Conclusions. The administration of penicillin in man results in the excretion of the substance by the liver in somewhat higher concentrations than are present in the blood stream. No penicillin could be demonstrated in the gastric juice.

Marjorie Jewell, Thelma Maxon and Grace Helm assisted in this work.

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[5] Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, to be published.

14292

Production of Pulmonary Edema by Thiourea in the Rat, and Its Relation to Age.*

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In earlier communications[1,2,3] we reported that the oral administration of certain sulfonamides, and of thiourea and its derivatives produces hyperplastic enlargement of the thyroid gland, concomitant with a marked drop in the B.M.R. in weanling and adult rats. Thyroidectomy cells were also found in the anterior pituitary. All of these changes were abolished by the parenteral administration of thyroxin. Iodine was of no avail in the prevention of this "thyroid-metabolic" effect. The hypothesis was advanced that the syndrome is due to an inhibition of thyroxin formation. A consequence of this inhibition is increased thyrotropic hormone formation by the pituitary with the resultant cellular stimulation of the thyroid. Astwood *et al.*[4] have published similar observations, and furthermore have met with success in the treatment of hyperthyroidism with thiourea and thiouracil.[5] Kennedy[6] reported that allyl thiourea will cause hyperplasia of the rat thyroid, together with changes in the cells of the anterior pituitary, an observation in agreement with our findings with respect to both thiourea and allyl thiourea.

Richter and Clisby[7] reported that 1-2 mg doses of phenyl thiourea usually proved to be lethal for their rats. Adult animals weighing between 164 and 350 g when given from 1-10 mg of this substance by various routes, showed signs of acute respiratory distress, accompanied by a marked drop in body temperature in less than 30 minutes. Their survival period varied from 2-18 hours. The only detectable pathologic signs were marked edema of the lungs and pleural effusion. These workers suggested that the depression in body temperature might be an indirect effect of the lowered body metabolism, resulting from the pleural effusion and respiratory distress. If the phenyl thiourea was administered in *sub-lethal doses* for a few days, and then gradually increased, the adult animals developed a tolerance for this substance. Adult rats, treated for several months in this

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* Supported by a grant from the Rockefeller Foundation.

[1] Mackenzie, J. B., Mackenzie, C. G., and McCollum, E. V., *Science*, 1941, **94**, 518.

[2] Mackenzie, J. B., and Mackenzie, C. G., *Federation Proc.*, 1942, **1**, 122.

[3] Mackenzie, C. G., and Mackenzie, J. B., *Endocrinology*, 1943, **32**, 185.

[4] Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinology*, 1943, **32**, 210.

[5] Astwood, E. B., *J. A. M. A.*, 1943, **122**, 74.

[6] Kennedy, T. H., *Nature*, 1942, **150**, 233.

[7] Richter, C. P., and Clisby, K. H., *Arch. Path.*, 1942, **33**, 46.

manner did not show any *thoracic changes* at autopsy; however, the thyroid glands were hyperplastic.

Binet[8] found that the subcutaneous injection of 600 mg of thiourea into a young 120 g rat resulted in a depression of body temperature, a slowing of the heart beat and a gradual collapse of the animal. Autopsy of this animal, which succumbed the following day, disclosed serous effusion in the pleura, unaccompanied by pulmonary congestion.

Hartzell[9] reported that the intravenous injection of 75 mg of thiourea into a rat (age and size not stated) caused the animal to die approximately 2 days later—the autopsy findings included passive congestion of the lungs, spleen and kidneys.

Our studies on thiourea at dietary levels of 0.05 to 2% were, with the exception of 2 adult animals, confined to weanling rats.[2,3] The 2 adult rats received the stock diet containing 1% thiourea for 2 months. After 2 weeks a definite depression in the B.M.R. was observed. Astwood[10] found that the daily oral administration of 2 g of thiourea to adult rats was not lethal over a period of 10 days.

In the course of subsequent experiments dealing with the effects of prolonged administration of sulfaguanidine on the thyroid gland of the rat, it was observed that these animals died with very striking symptoms when given for one night a diet containing 1% thiourea. Three out of 4 adult animals, previously fed the stock diet containing 2% sulfaguanidine for a period of 7½ months, when transferred to a diet containing 1% thiourea were found dead the following morning, while the fourth was moribund. The surviving animal showed signs of acute respiratory distress. The body temperature was below 92°F. Autopsy of this animal revealed the presence of 7 cc of clear, straw-colored serous fluid in the pleural cavity and marked areas of hemorrhage in the lungs. The fluid contained many cells and clotted rapidly on exposure to the air. The free drug level of sulfaguanidine in this animal was 8.4 mg %. The same condition was found in the 3 dead animals (Group A, Table I). Histologic examination of the lungs of our animals showed changes typical of those seen in pulmonary edema. The response of these adult rats to the oral ingestion of thiourea was identical to that observed by Richter and Clisby[7] in adult rats given phenyl thiourea.

In view of these findings, it seemed likely that the administration of thiourea to rats was incompatible with the administration of sulfaguanidine, possibly due to the formation of phenyl thiourea. This hypothesis was tested by feeding 2% sulfaguanidine and 0.5% thiourea simultaneously to young adult rats (5 months of age) and to immature animals (1¼-1½ months of age). As can be seen from Table I, in the former instance, 14 out of 16 animals died over night, while in the latter case only 2 out of 8 rats succumbed (Groups B and C). However, similar results were obtained with thiourea alone, indicating not only that it is the offending substance, but also that young adult animals (5 months of age) are very susceptible to its *toxic action*, as compared with immature rats (Groups D and F). Young adult rats taken from our stock colony,† and allowed access to the stock diet containing 1% thiourea were found dead the following morning. The pleural cavity contained fluid in amounts varying from 7-15 cc (Group D). Food consumption records revealed that as little as 5 mg of thiourea was sufficient to cause death in many young adult animals. In this group of animals, 2 females who consumed 3 and 4 g respectively of the diet (30 and 40 mg of thiourea) did not succumb. Autopsy of one of these animals revealed hemorrhagic sites in the lungs, but no pleural fluid. The other animal has been allowed to stay on the 1% thiourea diet and has survived.

Three 2-months-old female rats (Group E) consuming 10-40 mg of thiourea did not die overnight, but were found to have between 2.75 and 4 cc of serous fluid in the pleural

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[8] Binet, P., *Rev. Med. Suisse Romande*, 1893, **13**, 628.

[9] Hartzell, A., *Contrib. Boyce Thompson Inst.*, 1940, **11**, 249.

[10] Astwood, E. B., *J. Pharm. and Exp. Therap.*, 1943, **78**, 79.

† *Mus Norvegicus.*

TABLE I.
Effect of Parenteral and Oral Administration of Thiourea on the Rat and Its Relation to Age.

Group	No. of animals	Age, approximate, mo	Range of body wt, g	Addition to stock diet	Dose of thiourea, mg	Time to death, hr	% dying	Amt of fluid in pleural cavity, cc
A	3M 1F	8½	167-265	1% thiourea		<16	100	4.5-7
B	6M	5	320-393	2% sulfaguanidine + 0.5% thiourea		<16	100	5-8
	6M 4F	”	260-405	”		<16	80	5-13
C	6M 2F	1¼-1½	75-92	”		<16	25	2-3
D	14M	5	300-390	1% thiourea		<16	100	7-15
	6F	”	248-280	”	5-30	<16	66	9-14
E	3F	2	120-144	”	10-40	>24	0	2.75-4
F	4M 4F	<1	40-50	”	20-70	<16	0	—
G	4M	2½	220-230		27 mg intraperit.	<3	100	5.5-8
	4M	”	210-239		18 mg orally	Died within 2-3 hrs	100	3-5
	5M	”	215-220		18 mg intraperit.	”	100	4.5-6
H	5M	5	350-380		18 mg orally	Died bet. 2¾-3¾ hrs	100	6-9
	3M 3F	”	255-317		18 mg intraperit.	Died within 3-5 hrs	83	6-15.5
I	4F	5	212-316		50 mg per kilo body wt intraperit.	<16	50	12-15
	4F	”	267-300		100 mg per kilo body wt intraperit.	<4	100	6-9
J	2M 4F	1	51-59		18 mg orally		0	—
	6M 2F	”	51-57		72 mg intraperit.		0	—
	4M 4F	”	48-52		90 mg intraperit.		0	—
	4M 3F	”	45-57		180 mg intraperit.		0	—
	6M	”	50-55		180 mg intraperit. (twice in 4 hrs)		0	—
	5M 5F	1¼-1½	86-92		90 mg intraperit.		0	—

cavity when sacrificed the following morning. The higher tolerance of the weanling rat to thiourea was shown in Group F where no deaths took place, even though as great or greater amounts of this substance were consumed than in the case of older animals.

When thiourea[‡] was injected intraperitoneally, a single dose of 18 mg killed some young adult animals within a 2-hour period. Death was usually preceded by a convulsion. The oral route was found to be as effective as the intraperitoneal route (Groups G and

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[‡] C.P. product obtained from Pfanstiehl Chemical Company, Waukegan, Illinois. Melting point, 179.5°C (uncor.). Melting point after 3 recrystallizations, 179-180°C (uncor.). Nitrogen—% recovered: 98.8, 99.6 and 99.7. We are indebted to Harry Eisenberg for this analysis.

H). When 50 mg per kilo of body weight of thiourea was given intraperitoneally to 4 young adult females, 2 animals died in 16 hours and 2 survived and were killed at the end of 24 hours. Fluid was found in the pleural cavity in every case. Increasing the level of thiourea to 100 mg per kilo of body weight caused death preceded by convulsions in the 4 young adult animals tested (Group I).

The results with 35 weanling rats (1 month of age) and 10 rats (1¼-1½ months of age) were in striking contrast to those reported above. Weanling rats injected intraperitoneally with 72, 90 and 180 mg of thiourea survived. Following the intraperitoneal injection of 90 mg of thiourea (1 cc solution) or 180 mg (2 cc solution) the animals appeared to be acutely ill and comatose for several hours. However, despite the 180 mg dose which on the basis of body weight is roughly 50 times that required to kill a 250 g adult rat, the weanling animals survived and were bright and alert the following day. Six weanling rats given 360 mg in 2 divided doses in a 4-hour interval, survived this treatment; 2 of these sacrificed at the end of 6 hours still had some of the injected fluid in the peritoneal cavity. The lungs of these animals appeared to be somewhat redder than usual, but no fluid was found in the pleural cavity (Group J).

The results with weanling and immature rats confirm our earlier findings with respect to thiourea, for we not only fed thiourea in the diet up to a 2% level, but administered 110 mg daily parenterally to weanling rats.[3] In the case of the 2 adult rats fed thiourea at a 1% level in the diet and reported in a previous publication, we can attribute their survival to a high tolerance to thiourea. It is pertinent to point out here that a search of our autopsy records disclosed that both animals had areas of hemorrhage in their lungs.

Since thiourea causes pulmonary edema in the rat, and also causes hyperplasia of the thyroid gland, and a lowering of the B.M.R. the possibility existed that the thyroid was in some way involved in the acute pulmonary effect. Two adult thyroidectomized rats (3 months after operation and with B.M.R.'s indicating complete thyroidectomy) weighing 211 and 230 g were injected with 18 mg of thiourea intraperitoneally. Both animals showed acute respiratory distress within 30 minutes after injection, and died 24 hours later. The toxic response was similar in all respects to that seen in non-thyroidectomized animals, except for a longer period of survival. At autopsy no thyroid tissue was found in either animal. This eliminates the thyroid gland as being essential for the pulmonary reaction. Furthermore unpublished data have shown that thyroid enlargement and a decrease in the B.M.R. occurs in the absence of pulmonary edema or a marked depression of body temperature. Studies on the effect of thiourea on body temperature of young animals are in progress. However, there remains the possibility that the resistance of the immature rat to pulmonary edema is associated with the greater increase in size of the thyroid produced by thiourea in these animals. In view of Astwood's findings that adult animals can tolerate 2 g of thiourea orally per day, it is evident that either the *strain of rat* he employed possessed a high tolerance for this substance, or that the *basal diet* used exerted a protective action with respect to pulmonary edema.

Conclusions. Thiourea, in addition to effecting thyroid enlargement and a decline in the B.M.R., produces in appropriate doses, rapid and fatal pulmonary edema in adult rats. No necessary relation between these two reactions has been demonstrated. The immature rat is much less susceptible to the pulmonary edema and more susceptible to the thyroid enlargement produced by this compound. On a weight basis it tolerates 50 times the lethal dose for adult rats.

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Effects of Gramicidin and Tyrocidine on Pathogenic Protozoa and a Spirochete.

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Dubos observed that autolyzed cultures of a soil bacillus (*Bacillus brevis*) were highly injurious to other bacteria.[1] From *B. brevis* cultures was first isolated an active crude material, tyrothricin; the latter was then found to be composed of two substances, gramicidin and tyrocidine. While both of these are effective *in vitro* against bacteria in minute amounts, they differ very considerably in specificity. Notably, gramicidin affects almost only gram-positive bacteria, whereas tyrocidine is not so limited and kills bacilli without regard to their gram-staining affinities. Further information is available in the recent summary by Dubos and Hotchkiss.[2] The effectiveness of these substances on non-bacterial parasites has not been extensively studied.

The present paper reports the action of gramicidin and tyrocidine on the flagellates *Leishmania tropica, Trypanosoma cruzi,* and *Trypanosoma lewisi,* on the spirochete *Leptospira icterohemorrhagiae,* and upon *Bartonella bacilliformis.* These organisms were grown in pure culture in semi-solid rabbit serum and hemoglobin agar containing the desired amounts of gramicidin and tyrocidine, incubated at room temperature (*ca.* 20-21°C), and corked with rubber stoppers. In addition, the effect of tyrothricin upon *Trichomonas vaginalis* both *in vitro* and *in vivo* is reported. This last organism with associated favorable bacteria was cultivated in Johnson's medium[5] containing the test substance in the fluid portion, and incubated at 37°C. Johnson's medium (liver infusion agar slants covered with a mixture of human serum and modified Ringer's fluid) was chosen since in it no bacteria are required for continued growth of the flagellate. Dr. Paul A. Younge selected the cases of "trichomonas vaginitis" for study, directed all therapeutic trials, and from his results kindly furnished the information contained herein.†

Results. (I) The effects of gramicidin and tyrocidine upon the organisms grown in pure culture are given in Table I. Gramicidin exerted no apparent harmful effect against any of these in the concentrations employed. Yet, used upon susceptible bacteria, the same concentrations are notably injurious. Bacterial susceptibility, as pointed out, is correlated to a high degree with gram-positive staining. Certain results, however, indicate that other types of gramicidin sensitivity exist. The gram-negative meningococcus and gonococcus are affected by it. Results with mammalian cells have given similar results. Thus bovine spermatozoa are quickly rendered motionless by gramicidin.[4] Yet when stained, the tails of normal spermatozoa do not retain the violet at all, and while the heads do retain it somewhat, the reaction cannot be compared to the staining of gram-positive bacteria.‡ However, in so far as the present results are concerned, no atypical susceptibility was found and the general correlation between gram-positiveness and gramicidin sensitivity appears to extend to the protozoa and the leptospira tested.

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* Technical assistance rendered by Miss Rebecca Stickney was most helpful.

[1] Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 1.

[2] Dubos, R. J., and Hotchkiss, R. D., *Trans. and Studies Coll. Phys. Philadelphia*, 1942, **10**, 11.

[3] Dubos, R. J., Hotchkiss, R. D., and Coburn, A. F., *J. Biol. Chem.*, 1942, **146**, 421.

[4] Henle, G., and Zittle, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 193.

[5] Johnson, G., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 567.

† These studies were carried on at The Free Hospital for Women, Brookline, Mass.

‡ Personal communication from Dr. Henle.

TABLE I.
Growth in Culture Medium Containing Gramicidin and Tyrocidine.

		Gramicidin (γ per cc of medium)				Tyrocidine (γ per cc of medium)			
Organism	Control	0.005	0.05	0.5	1.0	0.1	0.5	1.0	5.0
Leishmania tropica	++++	++++	++++	++++	++++	++++	++++	++++	0 or +
Trypanosoma cruzi	++++	++++	++++	++++	++++	++++	++++	++++	+
Trypanosoma lewisi	++++	++++	++++	++++	++++	++++	++++	++++	+
Leptospira icterohemorrhagiæ	++++	++++	++++	++++	++++	++++	++++	++++	++++
Bartonella bacilliformis	++++	++++	++++	++++	++++	++++	++++	++++	++++

Tyrocidine, on the contrary, in a concentration of 5 γ per cc, proved to have a markedly adverse effect upon all the flagellates tested. This was evident by the slower rate of development, at times by the complete abolition of growth as in the case of *L. tropica,* as well as by less copious cultures.

Tyrocidine acted by killing the inoculated organisms. The effect was not instantaneous; the flagellates remained active for many hours, but after a lapse of several days had lost their motility. A few usually escaped and thus gave rise to the delayed growth.

Positive cultures of *Leishmania tropica* appeared normal in every respect, *Trypanosoma lewisi* colonies were somewhat more diffuse and at a deeper level, but were otherwise similar to the controls, whereas *Trypanosoma cruzi* cultures differed in both macroscopic and microscopic appearance.

Normally, in semi-solid medium, *T. cruzi* produces a thick, white, cloudy growth in the topmost centimeter of the medium, very scanty growth elsewhere. In the 5 γ tcd. (= 5 γ tyrocidine per cc) tubes, after an initial delay, growth appeared throughout the whole depth of the tubes as scattered white colonies reaching a relatively large size (0.2 to 0.3 mm). Those colonies which developed at a level lower than in the control tubes supported a certain degree of anaerobiasis, since in this medium methylene blue is decolorized to within about 1 cm of the surface.

This abnormal macroscopic growth corresponded to morphological peculiarities. In a normal culture of *T. cruzi,* long, thin, whiplike crithidial forms are numerous and prominent. They were extremely rare in the 5 γ tcd. cultures and instead one usually encountered crithidia with enlarged rounded posterior extremities. These stubby crithidia seemed healthy since they were actively motile, multiplied, and had a longevity fully equal to the normal elongate forms.

The second peculiarity consisted in the immaturity of the trypanosomes. Although in 40- to 60-day cultures the trypanosomes were not uncommon, in almost every instance they contained a rod-like relatively small kinetoplast, situated near the nucleus. In contrast, in normal cultures of equal age, trypanosomes resembling those found in the blood, that is to say, with a large round kinetoplast occurring near the posterior end, were far more frequent.

This variant growth which continued as long as the organism was maintained in the tyrocidine medium, was not a permanent modification, for upon successive transfers in normal, semi-solid medium, the cultures gradually reverted to a more typical appearance.

It seemed of interest to determine whether the dissemination of the colonies throughout the medium was correlated with a change in the metabolic requirements of *T. cruzi,* particularly in regard to growth under anaerobic conditions. To test this, organisms obtained from the bottom of 5 γ tcd. tubes were inoculated into semi-solid medium containing 0.1% of sodium thioglycollate. The organisms survived the oxygen deprivation, but colonies were scanty and small so that the result indicated survival rather than growth. Control anaerobic culture with organisms never treated with tyrocidine developed similarly. Therefore, there seemed to be no increased tolerance to reduced oxygen.

(II) *Trichomonas vaginalis* in culture was treated with varying concentrations of tyrothricin, which contains 10 to 20% gramicidin and 40 to 60% tyrocidine.[3] Concentrations of 50 γ per cc of culture fluid were invariably effective in killing the flagellates. It is probable that the effect was direct, rather than by the intermediary of the associated bacteria, since the medium employed supports growth of *T. vaginalis* in pure culture. It is also probable that the effective substance in the tyrothricin was the tyrocidine; gramicidin not being known to affect flagellates adversely.

Since these substances have been effectively employed in treating surface infections, it was thought that "trichomonas vaginitis" might be amenable to tyrothricin treatment. Accordingly, Dr. Paul Younge organized a small scale clinical trial. To avoid conjugal reinfection patients were requested to refrain from intercourse during treatment and all stated that they did so. Tyrothricin was used in a concentration of ½ mg (500 γ) per cc. The vagina was thoroughly scrubbed with this solution, and subsequently a vaginal tampon wet with the same solution was put in place and left for 24 hours.

The immediate effect was a dramatic disappearance of the trichomonads. After an interval of a few days, however, the organisms reappeared and the relapse forms were apparently uninfluenced by the same tyrothricin treatment which had previously been so effective. Several possibilities may explain this result: first, an acquired resistance to tyrothricin, and second, multiplication of those few trichomonads which may have been tyrothricin-resistant from the outset with destruction of the non-resistant forms. Also both of these possibilities apply to the associated vaginal bacteria which favorized the trichomonads.

Summary. 1. The effects of substances derived from *Bacillus brevis* were tested on protozoa, a leptospira, and on *Bartonella bacilliformis*. 2. Tyrocidine in a concentration of 5.0 γ per cc markedly inhibited the growth of *Leishmania tropica, Trypanosoma cruzi,* and *Trypanosoma lewisi* but not that of either *Leptospira icterohemorrhagiae* or *Bartonella bacilliformis*. 3. Tyrothricin killed *Trichomonas vaginalis* in culture at a concentration of 50 γ per cc of fluid medium. In patients with "trichomonas vaginitis" tyrothricin caused an initial dramatic disappearance of the trichomonads; relapse forms were uninfluenced by further applications. 4. Gramicidin in concentrations of 1.0 γ per cc of culture medium, or less, exerted no apparent adverse effect on the hemoflagellates nor upon *Leptospira icterohemorrhagiae* or *Bartonella bacilliformis*. These organisms are all gram-negative. The results obtained extend the correlation between gram-positive staining and gramicidin sensitivity.

14294

Oxygen Consumption of Regenerating Skeletal Muscle.

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In the process of neuromuscular regeneration, the onset of functional reinnervation initiates a rapid and complete restoration of glycogen concentration and a simultaneous but slower recovery of muscle mass and creatine concentration.[1] Because of these findings and the fact that many growing tissues exhibit high metabolic rates, it has seemed of interest to study the rate of oxygen consumption of regenerating muscle. For comparative purposes, the oxygen consumption *in vitro* of totally denervated muscle has also been reinvestigated.

Methods. The experiments were carried out on the soleus muscles of rats matched as to age, weight and stock. Complete denervation was accomplished by section of the tibial nerve. In a number of 110-day-old animals this nerve was crushed with a heavy ligature in order to permit reinnervation and subsequent muscle regeneration. Contralateral normal or denervated solei served as controls. The determinations were made at 37.5°C in Barcroft-Warburg manometers, using a phosphate-saline medium[2] and a 100% O_2 gas phase. Muscles from one experimental group were removed under light ether anesthesia while the remaining experiments were carried out on muscles from rats which had been killed by a blow on the head. To facilitate oxygen diffusion the muscles were split lengthwise into strips *in situ.* An average time interval of 35 minutes was consumed in preparation of the muscles, adjusting the apparatus and equilibrating for 15 minutes. The oxygen uptake for the next hour was then measured. The dry weights of the muscles, which ranged from 9 to 28 mg, served both for calculation of the O_2 consumption and estimating the degree of atrophy.

Results. Preliminary determinations on unoperated animals selected at random were made to ascertain the variances in O_2 con-

TABLE I.

Rate of Oxygen Consumption Together with Standard Errors for Normal, Totally Denervated, and Regenerating Soleus Muscles.

Experimental condition		No. of animals	Days after lesion		% muscle weight loss		Q_{O_2}		Q_{O_2}†
			L	R	L	R	L	R	
I	Unoperated	9	0	0	0	0	2.21 ± .13	2.30 ± .11	96.8 ± 5.9
II	Left tibial nerve cut	19	14	0	31.8	0	2.66 ± .14	2.40 ± .10	112.2 ± 4.98
III A	Left tibial nerve crushed	14	15-35	0	32.1 (20-50)	0	2.85	2.37 ± .11	122.3
B	Left tibial nerve crushed	13	18-42	0	8.5 (0-20)	0	2.49	2.76 ± .10	91.2
IV A	Left tibial nerve crushed; right tibial nerve cut	10	19	19	34.4*	45.0*	2.78 ± .19	2.82 ± .18	98.8 ± 1.9
B	Left tibial nerve crushed; right tibial nerve cut	6	20	13	25.1*	39.1*	2.65 ± .24	2.95 ± .12	90.6 ± 9.5

* Calculated on basis of normal dry muscle weight to body weight ratio of .0891%.

† Q_{O_2} of left muscle expressed as % of Q_{O_2} of right muscle.

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[1] Lazere, B., Thomson, J. D., and Hines, H. M., *Am. J. Physiol.*, 1943, **138**, 357.

[2] Hastings, A. B., Muus, J., and Bessey, O. A., *J. Biol. Chem.*, 1939, **129**, 295.

sumption between solei from different animals and also between the two solei of single animals (Table I, I).

The tibial nerves of 19 animals were cut and the oxygen consumption measured 14 days later. It was found that the O_2 consumption of denervated muscles was on the average slightly higher than that of their unoperated controls (Table I, II), the statistical probability of the observed increase being significant lying between 95% and 98%. The oxygen consumption following a unilateral nerve crush was measured at time intervals ranging from 15 to 42 days after denervation. When the results were arrayed according to the degree of atrophy it was noticed that the muscles exhibiting greater weight loss tended to possess slightly higher metabolic rates than their unoperated controls (Table I, III A and B). Calculation of the correlation coefficient between the percentage of weight loss and the gain in Q_{O_2} showed that only a fair correlation existed ($r = .52 \pm .14$). To compare the oxygen uptake of totally denervated muscle with that of regenerating muscle, the left tibial nerve was crushed and the right tibial cut in another series of 110-days-old rats (Table I, IV A and B). When the weight losses of the contralateral muscles were of the same order, no significant difference in O_2 consumption between the regenerating and the completely denervated muscles was detectable.

Conclusions. Although in some instances statistical interpretation of the data was limited by the smallness of the sample size in relation to size of the mean differences and standard deviations, it may be concluded with reasonable certainty that regenerating muscle exhibits a slightly increased rate of oxygen consumption *in vitro* per gram of tissue, but that the rate of oxygen consumption of regenerating muscle does not differ significantly from that of completely denervated muscle which has undergone a similar degree of weight loss.

14295

Histamine Ineffective in the Rat as a Gastric Secretory Stimulant.

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In the course of a comparative study of gastric secretion it was observed that the rat did not respond to histamine when given in doses found effective in other mammals. This seemed of sufficient interest to warrant special study since in our experience the only animals found refractory to histamine are the elasmobranch fishes[1] and the lower amphibia.[2]

Young albino rats, from 180 to 240 g body weight, kept on a diet of Purina dog chow, were used. Because of the well known habit of coprophagy, the stomach ordinarily is found to be filled with ingestia even after withholding food for 48 hours. By enclosing the rat in a specially constructed jacket, Roe and Dyer[3] were able to obtain animals with empty stomachs. We used this method but later abandoned it in favor of a simpler one. Keeping the animals without food in specially constructed wide-mesh, false-bottom cages proved satisfactory. Water was allowed *ad lib.* Only 5 to 12% of more than 1000 rats were found to have stomachs containing ingested matter after 24 to 36 hours' fast.

The animal was anesthetized with nem-

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[1] Babkin, B. P., Chaisson, A. F., and Friedman, M. H. F., *J. Biol. Board Canada*, 1934, **1**, 251.

[2] Friedman, M. H. F., *J. Cell. Comp. Physiol.*, 1942, **20**, 379.

[3] Roe, J. H., and Dyer, H. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 603.

TABLE I.

Effect of Histamine on Gastric Secretion in the Rat. Following a Control Period of 30 Minutes All Animals (Except Those in the Control Series) Were Given Histamine in the Doses Indicated.

	Control—65 rats			Histamine								
				0.02 mg per kg—37 rats			0.2 to 0.4 mg per kg—29 rats			3.5 to 5.5 mg per kg—34 rats		
		m. eq/l. acid			m. eq/l. acid			m. eq/l. acid			m. eq/l. acid	
Time	vol. cc	free	total	vol. cc	free	total	vol. cc	free	total	vol. cc	free	total
Control												
30 min	2.0	70	80	1.9	57	78	1.8	80	98	1.7	63	70
After histamine												
30 min	1.5	88	98	1.5	76	92	1.8	84	98	1.9	76	90
90 ”	1.4	64	81	1.5	61	79	1.2	71	90	1.7	88	101
150 ”	0.9	54	66	0.4	57	70	0.7	52	68	0.8	90	113
210 ”	0.7	50	54	0.6	55	68	0.8	50	60	0.4	70	86
Avg vol. for last 3½ hr	4.5	—	—	4.0	—	—	4.5	—	—	4.8	—	—
Max. avg acidity m. eq/l.	—	88	98	—	76	92	—	84	98	—	100	113
Total output of HCl during last 3½ hr, mg	—	11.13	13.05	—	9.53	11.86	—	11.39	13.87	—	14.38	17.06
Total output of acid as % of control group	—	100%	100%	—	85.6%	91.0%	—	102.3%	106.2%	—	129.0%	130.7%

butal or ether. In some instances gastric fistulas were established in the usual manner, but in most of the experiments samples of gastric juice were obtained by the method of Roe and Dyer.[3] The stomach was exposed through a small mid-line incision, the duodenum carefully ligated and a number 18 hypodermic needle inserted into the stomach in the region of the rumen. The needle served for withdrawing gastric contents. With care the stomach could be punctured repeatedly without contaminating the gastric contents with blood. Between periods of withdrawing gastric samples the abdomen was closed with small serrefines.

Histamine phosphate was given subcutaneously every 15 minutes, the first injection being made after an initial control period of 30 minutes. The doses used are shown in Table I. The acidities were determined with 0.01 N NaOH, using Topfer's reagent and phenolphthalein as indicators.

The results are summarized in Table I. The following conclusions may be drawn from the present study on 165 rats:

1. *The fasting rat continuously secretes small amounts of acid gastric juice.* While this secretion is greatest at the beginning, it still persists at a fairly steady rate during the 3rd and 4th hours and, as we have found in other experiments, even after 5 hours. Whether, as in the groundhog,[4] the continuous secretion is spontaneous and perhaps due to some innate properties of the secretory cells or, as in the dog,[5] the secretion is perhaps due to the presence of digestion products in the intestine, we do not know. In 12 rats, atropine in doses of 0.8 to 1.0 mg per kilo failed to inhibit this continuous secretion. The secretion probably was not due to operative trauma since further traumatizing the limbs and intestine 1 to 2 hours after the initial operation did not increase the secretion. That the gradual decrease in secretory rate from the initial high value was due to dehydration consequent to loss of gastric juice from the body[6] should be considered.

2. *Histamine does not stimulate in the rat an increased secretion of fluid.* The total

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[4] Friedman, M. H. F., and Armour, J. C., *J. Cell. Comp. Physiol.*, 1936, **8**, 201.

[5] Babkin, B. P., *Emanuel Libman Anniversary Volumes*, 1932, **1**, 11, International Press, New York.

[6] Friedman, M. H. F., *J. Cell. Comp. Physiol.*, 1939, **13**, 219.

volume of gastric fluid secreted after 3½ hours of histamine injections was equal to that of the control series during the same period. With extremely large doses (3.5 to 5.5 mg per kg) the secretion appeared to be concentrated with respect to acid. However, the response was not uniform and the acidity values in this group were widely distributed. Further study would be required to determine whether a dissociation between acid and water secretion really does occur in the rat as these results might suggest. These results do not confirm those of Roe and Dyer[3] who found that 0.03 mg of histamine phosphate per kilo does stimulate an acid secretion. The curves of both the volume and acidity of their histamine-treated rats parallel closely those we obtained in our series that received no histamine. Polland[7] found no free acid secreted by the rat after huge unstated doses of histamine.

With respect to the effect of histamine on the gastric secretory cells, the rat behaves like the skate[1] and necturus[2] rather than other rodents (groundhogs,[4] guinea pig).[8] The extremely high tolerance of the rat to histamine is well known.[9] The ineffectiveness of histamine in the rat as a secretory stimulant may perhaps be related to the rapid rate of disappearance of injected histamine from the blood and the relatively small amount of histamine retained by the stomach.[10] Since histamine is a potent gastric secretory stimulant in the anesthetized groundhog,[4] guinea pig,[8] cat,[11] dog,[12] pigeon[6] and chicken,[6] it is unlikely that the ineffectiveness of histamine in the rat was due in these experiments to the use of anesthetics.

Conclusion. In the rat gastric secretion is continuous. Histamine has little if any effect on the gastric secretion of the (anesthetized) rat.

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[7] Polland, W. S., cited by Bloomfield and Polland, *Gastric Anacidity*, Macmillan, New York, 1933, p. 80.

[8] Podolsky, H. M., and Friedman, M. H. F., in preparation for publication.

[9] Best, C. H., and McHenry, E. W., *Physiol. Rev.*, 1931, **11**, 371.

[10] Rose, B., and Brown, J. S. L., *Am. J. Physiol.*, 1938, **124**, 412.

[11] Noble, R. L., and Robertson, J. D., *J. Physiol.*, 1938, **93**, 430.

[12] Bowie, D. J., and Vineberg, A. M., *Quart. J. Exp. Physiol.*, 1935, **25**, 247.

14296 P

Return of Vision in Transplanted Adult Salamander Eyes After Several Days of Refrigeration.*

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It has been shown that adult salamander eyes can be transplanted successfully if they are grafted immediately after enucleation.[1,2] Return of vision 4 times in the same eye repeatedly transplanted demonstrates[3] how successful the results can be. The capacity of an adult salamander eye to recover is tested further in the following experiments by isolating it from its blood supply for a long period.

Fifty-nine enucleated adult *Triturus viridescens* eyes were refrigerated from 0°C to 8°C in sterile Ringer's solution for periods varying from 2 to 14 days and then transplanted into freshly denuded orbits of new

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* Aided by grants from the John and Mary R. Markle Foundation and the Fluid Research Fund of Yale University.

[1] Stone, L. S., and Zaur, I. S., *J. Exp. Zool.*, 1940, **85**, 243.

[2] Stone, L. S., and Cole, C. H., *Yale J. Biol. and Med.*, 1943, **15**, 735.

[3] Stone, L. S., and Farthing, T. E., *J. Exp. Zool.*, 1942, **91**, 265.

hosts. Under freezing conditions (0°C) the eyes were usually imbedded in solid ice in a little over an hour after they were placed in the refrigerator. The grafts exposed to the lowest temperature were first thawed in cool (10°C) Ringer's solution before transplantation. After 4 days these eyes are usually clear but the iris sinks inward with the pupil relaxed and distorted. By 7 and 8 days the cornea may be either slightly or densely opaque. In some cases, portions of the cornea, along with degenerating iris cells, begin to slough. The lens usually becomes opaque and degenerated, and by this time only rarely is it still clear. All of the grafted eyes which had been frozen showed little power for recovery. They sloughed or were rapidly resorbed so that by the second month they were merely small white sacs.

When the eyes were maintained at a higher temperature (8°C) in the ice box better results were obtained. At the end of 2 days at this temperature the eyes appeared about the same as at the time of excision. A few eyes transplanted at this time recovered and showed return of vision by the third month. Compared with eyes grafted immediately after excision recovery was slower in the survivals. The degeneration stages seemed to take place earlier and were so extensive, in some cases, that by the second month they were resorbed.

When kept in the ice box for 3 days at this temperature (8°C) more extensive injury to the cells was produced. The eyes still remained clear but the luster and color of the iris was not as brilliant as in the beginning of the experiment. The appearance of the iris indicated that degeneration was more rapid after transplantation. The cornea usually became cloudy but in some cases it was thick and clear enough to observe circulation in the iris as long as 86 days. Most eyes became small, darkly pigmented sacs by the end of the second month. Some eyes showed prolonged attempts to recover during the second and third months but all became gradually smaller and finally resorbed. When kept for 4, 5 and 6 days at 8°C the eyes became progressively softer and more extensively damaged. Although some eyes after transplantation varied slightly in the time at which they finally degenerated, the longer they were kept in the ice box the earlier the grafts began to break down and slough portions of the eyeball. Although one transplant which had been in the ice box for 4 days still had a semi-transparent cornea at 90 days, those grafts which had been refrigerated for 5 and 6 days had undergone considerable resorption by the fortieth day. During the first month some eye tissues such as the cornea and small portions of the iris often made short but futile attempts to regenerate.

The most ideal temperature for preserving the cells of the eye alive over longer periods was between 4°C and 6°C. The success after 3 days of refrigeration at this temperature is indicated by the fact that eyes in some cases had recovered vision at the end of 3 months. When kept for 4, 6 and 7 days, at this temperature the lens and cornea are nearly always clear and the iris, although paler in color, showed no degenerating cells on the surface. In nearly all of these grafts return of circulation was clearly seen. Some cases resorbed slowly but not without an attempt to recover. In 2 cases, for example, where the eyes had remained in the ice box for 7 days, the pigment pattern of the iris was attempting to reform by the fortieth day, and newly regenerated lenses had formed from the dorsal iris. These eyes are still progressively recovering during the second month.

At the end of 10 days in the ice box at this temperature the cornea still remains clear but the lens begins to take on an opaque appearance. The iris is about the same as at the end of a week. By the 14th day the chief change consists of the complete breakdown of the lens and the general softness of the eyeball. When transplanted at this time many loose cells appear under the clear cornea indicating how easily the cells of the interior of the bulb can be dislodged by the manipulations during operation. These eyes break down rapidly during the first 3 weeks after transplantation. The cornea may, however, remain partly clear for this period. Remnants of it and the sclera are about the last to resorb.

14297

The Internal Biliary Fistula Dog Unsuitable as a Bioassay Animal for Liver Extract.*

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From the Department of Pharmacology, The University of Chicago.

Crandall, Finne, and Smith[1] reported a pernicious anemia-like state in dogs with internal biliary fistulae. This anemia was described as macrocytic and hyperchromic, characterized by spontaneous remissions with reticulocyte showers. Single intramuscular injections of 30 U.S.P. units of liver extract in 6 animals resulted in reticulocyte increases of 2-20% and a return of mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCHb) to within 2 standard deviations of the normal mean. Frequent injections totaling more than 30 units resulted in increases in the red blood count of a million or more cells. The present study is an attempt to confirm the work of Crandall's group and to evaluate its potentialities as an experimental bioassay method for the antipernicious anemia principle in liver extract.

Standard internal biliary fistulae were produced in dogs by suturing the gall bladder into the right renal pelvis; the common bile duct was ligated and sectioned. U. S. Bureau of Standards equipment was used in the erythrocyte counts. Hemoglobin (Newcomer method), reticulocyte (wet method), and hematocrit (Van Allen) determinations were also made. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCHb) were calculated. Plasma bilirubin, chloride, non-protein nitrogen, and protein determinations were made at intervals during the experimental period. All animals were autopsied and examined for possible patent bile duct connections to the intestine. Histological sections of bone marrow and other tissues were prepared.

The dogs were fed a standard diet of canned dog food. Fat soluble vitamins[†] were administered parenterally in adequate doses throughout the experimental period.

Results. A mild anemia appeared in 4 out of 7 dogs approximately 4-6 months after operation; data on one of these dogs were discarded since autopsy revealed a patent probably anomalous duct between the gall bladder and intestine. The anemia which appeared may be described as mildly macrocytic and irregularly hyperchromic. The anemia in all dogs was characterized by spontaneous remissions and relapses (Fig. 1) not associated with reticulocyte showers.

Blood chemistry values including the Van den Bergh Reaction were essentially normal during the post-operative period.

Three animals were given synthetic vitamins of the B complex intravenously in excess of the required daily dose for a period of one week. This was done to make certain that the animals were not deficient in these vitamins before testing for a response to liver extract. No hematopoietic response was observed.

In order to test for a hematopoietic response to liver extract, two clinically standardized preparations[‡] (15 U.S.P. units/cc) were administered parenterally to 3 dogs in doses totaling 30-60 units.

No statistically significant hematological responses were observed over and above the anemic level. Fig. 2 indicates the lack of response in one of the dogs.

Bone marrow sections did not reveal the megaloblastic bone marrow characteristic of pernicious anemia.

Discussion. The variation in the anemic

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* The investigation was aided by a grant from the Armour Research Fund of the University of Chicago.

[1] Crandall, L. A., Finne, C. O., and Smith, P. W., *Science*, 1941, **93**, 549.

[†] Ephynal acetate obtained through the kindness of Hoffmann-La Roche, Inc.

[‡] Armour Liver Extract (generously furnished by Armour Laboratories); Lilly Liver Extract, Purified.

FIGURE 1. SPONTANEOUS REMISSIONS (ACE)

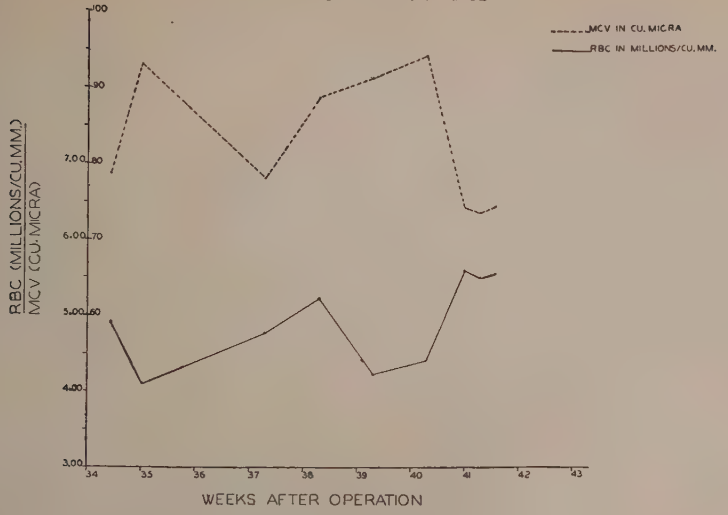
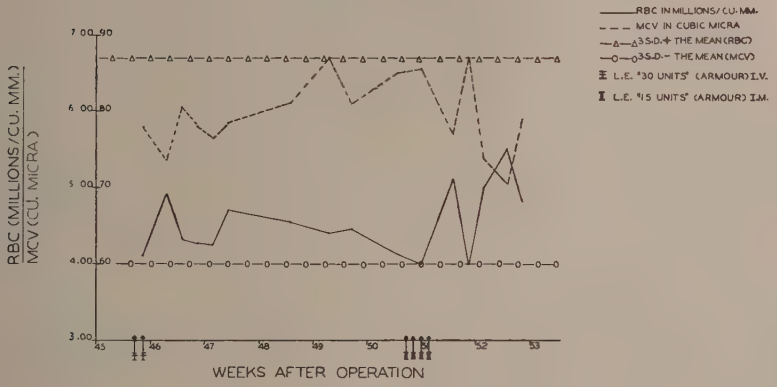


FIGURE 2. TEST FOR RESPONSE TO LIVER EXTRACT (ACE)



level was such that 3 Standard Deviations ± the mean yielded values above the normal R.B.C. and below the normal MCV. Under such conditions it would be impossible to obtain a statistically significant response. This fact is a practical objection to the method if a response is to be judged in reference to the variation in values obtained during the non-treatment period of the anemia. Since neither a significant reticulocyte response nor a maintained rise in the red blood count was shown by the dogs receiving liver therapy, it seems unlikely that any response took place. The absence of a pernicious anemia-like bone marrow helps to explain the lack of a response of the peripheral blood picture to liver therapy.

Crandall's criterion of a response is the return of the anemic MCV and MCHb to within 2 S.D. of the normal mean without reference to the degree of variation in these values during the anemic period. Although changes in MCV and MCHb took place in his animals following liver therapy, there were no clear cut responses in red blood cell and reticulocyte counts. In view of the spontaneous remissions in the anemic blood picture, it would seem that definite and consistent reticulocyte and erythrocyte increases should be included in the criteria to assure the existence of a response.

A definite hematological response should be obvious in reference to either the anemic level or the normal blood picture. The spontaneous variations during the anemic period and the indefinite nature of reticulocyte and red blood cell increases following liver therapy makes this method impractical as a means of bioassay.

Summary and Conclusions. 1. Internal biliary fistulae were produced in dogs. 2. A low grade anemia, mildly macrocytic and irregularly hyperchromic appeared. 3. The parenteral administration of clinically standardized liver extracts elicited no hematopoietic response. 4. This approach is of no value as a bioassay procedure for the Antipernicious Anemia Principle in liver extract.

We wish to express our appreciation to Dr. Raphael Isaacs for helpful suggestions and criticisms. Acknowledgment is made to Dr. Clarence Lushbaugh of the Department of Pathology for the preparation and analysis of histological sections, to Dr. Willard Freeman of the Department of Physiology for his coöperation on the operative procedures, and to Mrs. Elizabeth Lewis of the Department of Pharmacology for the blood chemistry analyses.

14298

Action of Bacterial Toxins on Tumors. V. Immunological Protection Against Tumor Hemorrhage.

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We have found, as similarly reported for *Salmonella typhi*,[1,2] *Salmonella typhimurium*[3] and *Shigella dysenteriae*[4] that the endotoxin of *Shigella paradysenteriae* Flexner when repeatedly administered parenterally to mice confers a definite although limited protection (4 to 6 LD50)* against subsequent doses of the toxin. The object of the present study was to determine whether tumors transplanted into mice previously immunized in the above manner are immune to hemorrhage following injection of Flexner endotoxin in amounts which regularly produce hemorrhage in tumors carried by non-immunized animals. A simultaneous protection against both lethality and tumor-hemorrhage conferred by previous immunizing treatment with the bacterial product would provide an independent test of the hypothesis elaborated in earlier papers of this series, to the effect that lethality as well as tumor-hemorrhage production are both manifestations of the vascular damage characteristic of the endotoxins of gram negative bacteria.[5,6,7]

White Rockland mice were immunized with successive doses of a *Shigella paradysenteriae* endotoxin preparation. Doses of one-half to an LD50 were injected intraperitoneally on 5 successive days, followed on the sixth day by a dose of one LD50. The endotoxin was prepared by growing *Shigella para-*

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[1] Favorite, G. O., and Morgan, H. R., *J. Clin. Invest.*, 1942, **21**, 589.

[2] Boivin, A., and Mesrobeanu, L., *C. R. Soc. Biol.*, 1938, **128**, 835.

[3] Zahl, Paul A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 116.

[4] Boivin, A., and Mesrobeanu, L., *C. R. Soc. Biol.*, 1938, **128**, 447.

* In conformity with current practice, we have abandoned the expression ''MLD'' in favor of the term ''LD50'' as indicating the dose lethal within 24 hours to 50% of the animals. Likewise, the term ''HD50'' indicates the dose which will produce definite tumor hemorrhage in 50% of the test animals.

[5] Zahl, Paul A., Hutner, S. H., Spitz S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224.

[6] Zahl, Paul A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 285.

[7] Hutner, S. H., and Zahl, Paul A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 364.

TABLE I.

Tumor Hemorrhage in Immunized and Non-Immunized Mice.

Amt of toxic material inj.	Immunized tumor-bearing mice (degree of hemorrhage)*	Non-immunized tumor-bearing mice (degree of hemorrhage)*
LD50/400†	0 0 0 0 0 0 0	0 0 0 0 0 0
LD50/200	0 0 0 0 0 0 0 0	0 0 0 0 0 ++ + + +
LD50/80	0 0 0 0 0 0 0	+++ +++ +++ ++ ++ ++ + +
LD50/40	0 0 0 0 0 0 0 0 0	+++ +++ +++ +++ ++ ++ ++ ++ ++
LD50/4	0 0 0 0 0 0 0 0 0 0	+++ +++ +++ +++ ++ ++ +
1 LD50	0 0 0 0 0 0 + + + + ++	+++ +++ +++ +++ +++ ++ ++ ++ + +
4 LD50‡	+++ +++ +++ +++ +++ ++ ++ ++ ++ ++	+++ +++ +++ +++ ++ ++ ++ ++ ++
6 LD50‡	+++ +++ +++ +++ +++ ++ ++ ++ ++	+++ +++ +++ +++ +++ +++ ++ ++ ++

Degree of hemorrhage:

0 = no hemorrhage
+ ± slight hemorrhage
++ = considerable hemorrhage
+++ = marked hemorrhage

* Each designation below represents one animal.

† The amount of dry toxic dysentery material necessary to produce death within 24 hours of 50% of non-immunized mice injected intraperitoneally was 10 mg. This is therefore designated as one LD50. Four normal LD50 or 40 mg of the toxic material was necessary to produce death in 50% of the immunized mice.

‡ Non-immunized animals at these dosages all succumbed to the lethal effects of the toxic material, but hemorrhage of the tumor was evident before death.

dysenteriae Flexner in liquid medium.† The identity and smoothness of the culture was confirmed by agglutination tests against a standard immune typing serum. After suitable growth the cultures were killed with phenol and the volume reduced by pervaporation through cellulose tubing, followed by precipitation with acetone according to the procedure previously described.[3] Animals immunized with this material were able to survive 4 to 6 LD50 doses of the same preparation on the tenth day after the first immunizing injection.

Ten to 15 days following the first injection, Sarcoma 180 was implanted into mice, using the standard trocar technic. Animals bearing 7-day-old tumors, together with a parallel control series of non-immunized tumor-bearing mice, were given intraperitoneal doses of the same Flexner preparation used for the immunization. Six hours after injection the tumors were examined and graded as to degree of hemorrhage. The results are listed in Table I. It is seen that in the control (non-immunized) animals hemorrhage was induced at all doses greater than 1/200 of an LD50. This 1:200 ratio between the lowest effective hemorrhage and lethal doses is almost identical to the ratio found to hold for the unpurified acetone precipitate of *Salmonella typhimurium* previously reported.[3] ‡ For the immunized animals the ratio between HD50 and LD50 is in the order of 1:1, all doses smaller than one LD50 failing to produce hemorrhage. It is evident that immunization with the endotoxin preparation used protects against both tumor-hemorrhage induction and primary lethality; also that the relative effectiveness of immunization is very much greater in the case of hemorrhage induction than in the case of primary lethality.

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† In addition to the usual inorganic salts, the medium contained:

(1) Nicotinic acid—0.04 mg%
(2) Na_3 citrate · $2H_2O$—0.1%
(3) Dextrose—2% (added aseptically)
(4) Casein acid hydrolysate diffusate—0.8%

The diffusate was prepared by dialyzing through cellulose tubing neutralized H_2SO_4-casein hydrolysate. The medium was devoid of high molecular weight compounds, and was therefore considered to be antigen-free.

After immunization the HD50 of the antigen is of about the same magnitude as the LD50, yet for the non-immunized tumor-bearing animals the ratio between HD50 and LD50 is 1:200. Hence it would seem that the neutralizing action of the antibody must be prompt and complete, since but a very small quantity of uncombined antigen would be sufficient to induce tumor hemorrhage. In this respect the protection provided by immunization appears to differ from that afforded by sulfanilamide in that immunization protects against two hundred HD50, whereas sulfanilamide protects against only sixteen HD50.[6] Evidently, and as one would suppose, the mode of antitoxic action of sulfanilamide is of a different nature from the immunological mode of protection.

The preceding papers of this series suggest a uniformity in constitution and in toxic action of the complex O antigens of nearly all gram negative bacteria. The widespread occurrence among gram negative bacteria of the tumor-hemorrhage incitant and its correlation with the occurrence of O antigens was adduced as evidence for the existence of a single uniform toxin in the O antigens of gram negative bacteria.

The present paper describes the type of antitoxic immunity obtained when the same organism is used for both immunization and hemorrhage production. Cross-immunization experiments, using gram negative organisms unrelated except for the exhibition of common tumor hemorrhage production—a highly sensitive method of detecting these endotoxins—is expected to furnish critical evidence as to the unitary character of these toxins. Such experiments are now in progress.

Conclusion. Tumors implanted in mice previously immunized with *Shigella paradysenteriae* Flexner were found, in contrast to those implanted in non-immunized control mice, to be highly resistant to the tumor-hemorrhage factor of the Flexner organism.

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‡ It is to be noted that in the case of highly purified or possibly dissociated antigen the HD50/LD50 dosage ratio may be as great as 1:5000.[3,8]

[8] Shear, M. J., *Cancer Research*, 1941, **1**, 731.

14299

Activity of Parathyroid Hormone in the Nephrectomized Rat.

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The question of whether some of the effects of parathyroid extract injections are producible in the absence of the kidneys has been studied by several investigators, in the effort to decide if parathormone acts primarily upon the kidneys.

Collip and others[1] found that the injection of 80 units of PTH into bilaterally nephrectomized rats produced the customary changes in the bones.

This has been confirmed and the observation recently extended by Selye[2] who showed that nephrectomy alone produces a mild degree of "osteitis fibrosa" which was prevented by parathyroidectomy.

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[1] Collip, J. B., Pugsley, L. I., Selye, H., and Thomson, D. L., *Brit. J. Exp. Path.*, 1934, **15**, 335.

[2] Selye, H., *Arch. Path.*, 1942, **34**, 625.

Ellsworth[3] observed an elevation of the serum Ca after injection of 700 units of PTH into nephrectomized dogs.

Tweedy and his coworkers[4,5] also working on nephrectomized dogs, could not detect a significant rise in serum Ca after injection of 100-250 units of PTH.

Recently Neufeld and Collip[6] reported that they were unable to obtain an elevation of the serum Ca after PTH injections into nephrectomized and ureter ligated rats, cats and dogs. There was, however, no comparison made with nephrectomized uninjected animals.

The difficulty in raising the serum Ca in animals deprived of their kidneys, is obvious. Considering the well known Ca-P antagonism and the fact that the serum phosphate in these animals is found to be several times higher than normal, it cannot be expected that animals without kidneys would respond like normal animals to PTH injections. Even in comparing injected and uninjected nephrectomized animals, there is still a possibility of misinterpretation. The fact that renal insufficiency leads to parathyroid hyperfunction is well known[7] and one may expect that in the uninjected nephrectomized animals used for comparison the parathyroids are hyperactive. Since the effect of PTH injections on the activity of the parathyroids is unknown, a second variable may be introduced when the experimental findings in the nephrectomized, PTH injected animals are compared with those in the uninjected nephrectomized controls. On reinvestigating the problem we wished to avoid this possibly improper comparison by removing both kidneys and parathyroids in the PTH injected and uninjected group. We also attempted to detect the extent of a possible parathyroid hyperactivity in the nephrectomized rat by its action on the serum Ca concentration.

Experiments. For our experimental series, 47 male albino rats were selected from our stock colony and divided into 9 groups of 5 to 6, using litter mates when possible. They ranged in age from 15-17 weeks and in body weight from 280-350 g. The animals were kept on our stock diet (Rockland pellets). Ether anesthesia was used for all operations and for killing the animals at the end of the experiments. One group of 6 animals served as normal controls, 6 were parathyroidectomized, 5 normal unoperated rats were injected with 5×20 units of PTH (Lilly extract) subcutaneously.* Two groups of 5 animals were nephrectomized; one group was continued on the stock diet, in the other food was withdrawn after the operation. Two groups of 5 animals were nephrectomized and immediately afterwards, parathyroidectomized, one group fasted after the operation. Two further groups of 5 parathyroidectomized-nephrectomized animals under the same conditions as the previous groups were injected subcutaneously with 5×20 units of PTH. All animals were killed 48 hours after the operations and first injections. The last injection was given 4-6 hours before the animals were killed. Blood was then taken from the right auricle and the serum analyzed in individual samples for Ca (Clark-Collip) and phosphates (Kuttner-Cohen). The total serum protein was determined in the nephrectomized groups with the refractometer. Bones were examined histologically.

Findings. The results are given in the table. The nephrectomized fasted and non-fasted groups were combined since no significant differences in the serum levels were found. The individual serum Ca values in the 3 nephrectomized groups are seen in the scatter chart. The experimental findings in the table have been arranged in 2 sections. In the first section is shown the effect of parathyroidectomy after 48 hours upon the serum Ca and phosphate in non-nephrectomized and nephrectomized animals. The second

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[3] Ellsworth, R., and Futcher, P. H., *Bull. Johns Hopkins Hosp.*, 1935, **57**, 91.

[4] Tweedy, W. R., Templeton, R. D., and McJunkin, F. A., *Am. J. Physiol.*, 1936, **115**, 514.

[5] Tweedy, W. R., *Endocr.*, 1937, **21**, 55.

[6] Neufeld, A. H., and Collip, J. B., *Endocr.*, 1942, **30**, 135.

[7] Pappenheimer, A. M., *J. Exp. Med.*, 1936, **64**, 965.

* We are greatly indebted to the Ely Lilly Company for the generous supply of parathyroid extract.

TABLE I.

	No. of animals	Serum Ca Avg	Serum Ca Range	Inorganic serum PO_4 Avg	Inorganic serum PO_4 Range
I *Non-nephrectomized*					
Normal control	6	10.7	10.3-11.0	7.7	7.3-8.2
Parathyroidectomized	6	8.3	6.6-9.7	9.0	8.0-9.5
Nephrectomized					
Non-parathyroidectomized	10	9.6	8.6-11.0	14.6	10.1-17.1
Parathyroidectomized	10	6.5	5.3-7.1	14.5	12.1-18.1
II *Non-nephrectomized*					
Normal control	6*	10.7	10.3-11.0	7.7	7.3-8.2
Injected	5	12.3	11.9-12.5	8.3	7.9-8.7
Nephrectomized					
Parathyroidectomized control	10*	6.5	5.3-7.1	14.5	12.1-18.1
Parathyroidectomized injected	10	10.5	8.8-11.9	15.3	14.1-16.9

* Same animals as in first section.

section serves the purpose of comparing the effect of PTH injections in the normal and double operated rats. It is seen from the data that after 48 hours parathyroidectomy alone lowers the serum Ca from 10.7 to 8.3 mg %. It increases the serum phosphate from 7.7 to 9.0 mg %. In the nephrectomized groups the serum Ca is 3.1 mg % lower after the combined operation than in those in which only the kidneys were removed. The serum phosphate values show no differences between the two groups. This clearly indicates that the parathyroids are able to maintain a relatively high blood Ca level in absence of the kidneys. The first 2 groups in the second section of the table demonstrate the well known serum Ca elevating effect of PTH injections in non-nephrectomized animals. The injection of 5×20 units of PTH produces after 48 hours an average rise in the serum Ca of 1.5 mg % without affecting the serum phosphate. The latter finding is in accordance with Collip's data. In the absence of the kidneys and parathyroids the same dosage produces an elevation of the serum Ca to 10.5 mg % compared with 6.5 mg % in the uninjected controls. The serum phosphate again is unchanged.

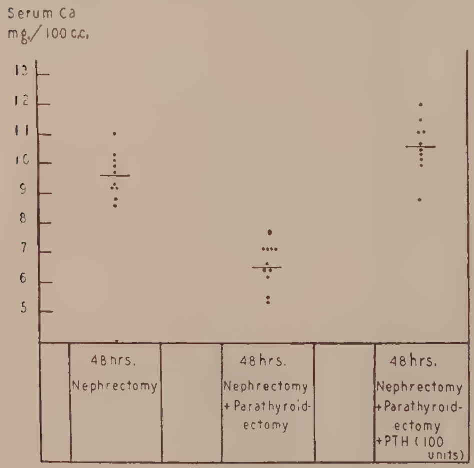

FIG. 1.

Thus in the absence of the kidneys, the blood Ca is maintained at a normal level because of the increased activity of the intact parathyroids; when these are removed, the blood Ca can be elevated by the injection of PTH.

The average total serum protein did not significantly differ within the 3 nephrectomized groups; it averaged 6.61 mg % in the nephrectomized animals, 5.91 mg % in the double operated and 6.22 mg % in the double operated injected group. Thus hemo-dilution or hemoconcentration is not likely to account for the differences in the blood Ca level. The histology of the bone, comparing the distal epiphysis of the right femur, was in accordance with the findings of Selye. "Osteitis fibrosa" was absent in the double operated uninjected group, mild in the nephrectomized and pronounced in the double operated injected animals.

Summary. Within 48 hours, parathyroidectomy lowers the serum Ca in non-nephrectomized mature rats from 10.7 to 8.3 mg %. In the same period of time the serum Ca in nephrectomized-parathyroidectomized rats decreases from 9.6 to 6.5 mg %.

The injection of 5×20 units of PTH into normal rats, after 48 hours produces an average elevation of the serum Ca of 1.5 mg %. The injection of the same quantity of PTH into parathyroidectomized-nephrectomized rats, after the same time produces an average elevation of the serum Ca of 4 mg % (from 6.5-10.5 mg %). Although these experiments prove that PTH raises the blood Ca without the intermediation of the kidneys, they do not exclude the possibility that the hormone may under normal conditions also affect renal function.

Conclusion. (1) The serum Ca elevating activity of the parathyroid hormone is demonstrable in bilaterally nephrectomized rats. (2) Confirming previous observations, it has been found that "osteitis fibrosa" is produced by PTH injection in the absence of the kidneys.

The author wishes to express his thanks to Mrs. Claudia Schogoleff for her technical assistance.

Since this paper was submitted, there has appeared a paper by Ingalls, Donaldson and Albright (*J. Clin. Invest.*, 1943, **22**, 4) showing that injections of PTH in nephrectomized rats produces more severe skeletal lesions than those which result from nephrectomy alone.

14300

B Vitamins in Germinating Seeds.*

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In the course of studies on the B vitamin contents of foods, it was noticed that samples of fresh corn, peas and beans contained greater amounts of certain B vitamins than did the dried seeds. This suggested that the B vitamin levels in these seeds might be related to their metabolic activity.

Germination of various seeds is known to be accompanied by increases in ascorbic acid and thiamin content.[1-4] Experiments were therefore begun to determine whether this was also true for the other B vitamins.

Samples of blackeyed peas, lima beans and cotton seeds were allowed to germinate in covered Petri dishes between layers of moist filter paper. The temperature was maintained at approximately 30° for periods of 36 and 48 hours. At the end of the germination period the seeds were ground, digested with takadiastase and papain according to the general enzyme procedure of Cheldelin, Eppright, Snell and Guirard,[5] and assayed for 8 B vitamins. Microbiological methods of assay were employed throughout.[6] The results are shown in Table I.

Comparisons are made on the dry weight basis, in order to compensate for differences in moisture content. Although many samples which absorb large amounts of water during germination do not appear to gain in

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[*] Presented before the Division of Biological Chemistry at the 104th meeting of the American Chemical Society, Buffalo, N.Y., September, 1942.

[†] Present address, Department of Chemistry, Oregon State College, Corvallis, Oregon.

[1] Biswas, H. G., and Das, K. L., *Science and Culture*, 1938, **4**, 360.

[2] Muthanna, M. C., and Ahmad, B., *Current Sci.*, 1940, **9**, 320.

[3] Mentzer, C., *Bull. soc. chim. biol.*, 1940, **22**, 444.

[4] Rytz, W., Jr., *C. R. Soc. Biol.*, 1938, **129**, 814.

[5] Cheldelin, V. H., Eppright, M. A., Snell, E. E., and Guirard, B. M., *The University of Texas Publication*, 1942, No. 4237, 15.

[6] Williams, R. J., *The University of Texas Publication*, 1941, No. 4137, 7.

TABLE I.
B Vitamin Content of Seeds.
(γ/g on dry weight basis.)

	Blackeyed Peas					Lima Beans					Cotton Seed		
	Content			Increased %		Content			Increased %				
	Before germination	After germination 36 hr	After germination 48 hr	36 hr	48 hr	Before germination	After germination 36 hr	After germination 48 hr	36 hr	48 hr	Before germ.	After germ. 36 hr	% Inc.
Dry wt, %	95	36	43			92	49	41			84	53	
Contents:													
Thiamin	8.5	12	11	41	30	5.8	4.7	4.8	—19	17	3.2	4.9	53
Riboflavin	1.5	3.9	3.9	160	160	1.4	2.4	2.4	71	71	2.3	2.8	22
Nicotinic Acid	14	24	51	71	260	11	16	26	46	140	16	24	50
Pantothenic Acid	11	13	17	18	55	9.0	11	20	22	122	11	22	100
Pyridoxin	2.0	3.3	4.6	65	130	6.0	6.7	9.5	12	58	0.91	2.6	190
Biotin	0.22	0.31	0.41	41	86	0.11	0.16	0.20	46	82	0.29	0.28	— 3
Inositol	2600	4400	4600	69	77	1800	3100	3800	72	110	3400	2800	—18
Folic Acid	7.8	4.6	4.2	—41	—46	3.6	2.7	4.2	—25	17	3.8	7.9	110

vitamin content, actually they are found to increase appreciably when only dry weights are considered.

It is apparent from Table I that the effect of germination on vitamin levels varies considerably for the different vitamins studied. A large increase in nicotinic acid is recorded upon germination. Riboflavin, pantothenic acid, pyridoxin, biotin and inositol also show appreciable increases in at least 2 of the seeds tested. Thiamin is a borderline case; it increases somewhat in peas and cotton but decreases in lima beans. Folic acid is seen to decrease considerably in peas, but there is a marked increase of this vitamin during the germination of cotton seed.‡

The increases in the rates of synthesis of the various vitamins also differ. Thus, thiamin and riboflavin do not appear to be synthesized in peas or beans after 36 hours of germination; the amounts present remain constant. The inositol contents also appear to be approaching a maximum after 48 hours. Nicotinic acid, pantothenic acid and pyridoxin, on the other hand, seem to be synthesized more slowly at first, but increase markedly between 36 and 48 hours. Biotin is intermediate.

The B vitamin levels of corn and beans at different stages of development have also been determined. The results are shown in Table II. The samples were taken from neighboring plants of the same age. The seeds were divided into 4 age groups representing progressive stages of growth. These are characterized in Table II according to the moisture content, the size of the ears of corn and bean pods. The age groups are designated as Age 1, 2, 3 and 4, respectively.

It is of interest to note that the percent of solids is less in the second age group than in the first. The reason for this is not apparent.

With both corn and beans most of the vitamins reach a concentration peak at the

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‡ Since this manuscript was originally prepared a study similar to the present one has been reported by Burkholder.[7] The results, insofar as they are comparable, are in generally good agreement with our own.

[7] Burkholder, P., *Science*, 1943, **97**, 562.

TABLE II.
B Vitamins in Corn and Beans at Different Stages of Development.
(γ/g on dry weight basis.)

	Corn (Golden Bantam)				Beans (Bush Lima)			
	Age I	Age II	Age III	Age IV	Age I	Age II	Age III	Age IV (Seeds)
Dry weight, %	15	7.8	26	70	31	21	82	92
No. specimens sampled	2 ears	3 ears	2 ears	1 ear	6 pods	6 pods	6 pods	1 lb
Avg wt per ear, g	5.7	26	87					
Wt corn per ear, g	3.6	12	31					
Length of pods, inches					1-1½	3	3	
Contents:								
Riboflavin	3.9	5.5	4.0	1.5	2.2	4.1	1.4	1.4
Nicotinic Acid	59	94	59	17	28	42	17	11
Pantothenic Acid	20	17	12		25	40	35	9.0
Pyridoxin	2.9	3.7	1.4	7.6	1.7	3.0	2.7	6.0
Biotin	1.3	1.4	0.35	0.21	0.25	0.39	0.41	0.11
Inositol	1500	2900	1500	1500	2000	4100	1600	1800
Folic Acid	5.0	7.9	2.8	1.6	13	18	5.7	3.6

second age of development, after which they decline steadily as the seeds mature. Pantothenic acid is seen to decline from the start in corn.

Pyridoxin is exceptional in that it is present in the highest concentration in the mature seeds. It is possible that pyridoxin may be a storage form of this vitamin and that pseudopyridoxin[8] may be the active form in the young plant.

Summary. Germination of seeds is accompanied by increases in the content of most B vitamins. The increases are greatest for nicotinic acid, followed by pyridoxin, pantothenic acid, riboflavin, inositol and biotin in approximately the order named. Thiamin is a borderline case. Folic acid is seen to decrease substantially in peas and in one sample of lima beans.

Contents of B vitamins in growing corn and beans are found to reach a peak at a relatively early stage of development, after which the amounts present decrease steadily as the seeds mature.

We wish to acknowledge, with thanks, the cooperation of Dr. Roger J. Williams, in whose laboratory this work was carried out. We are also indebted to Research Corporation of New York for financial support.

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[8] Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

14301

Pantoyltaurine and Growth of Rats.

KLAUS UNNA.

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It has been shown by Snell[1] and by McIlwain[2] that N-(a-γ-dihydroxy-β-β-dimethyl butyryl Taurine (pantoyltaurine)) inhibits the growth of certain bacteria which require pantothenic acid as an essential nutrient. The inhibition was reversed by the addition of large amounts of pantothenic acid. In analogy to the relationship between pyridine-3-sulfonic acid and nicotinic acid[3] it was assumed by these authors that by virtue of its structural similarity pantoyltaurine competes with the utilization of pantothenic acid, particularly since the growth of bacteria which do not require pantothenic acid was not inhibited by pantoyltaurine. In connection with earlier studies on the utilization of pantothenic acid derivatives in bacteria and in animals[4] we became interested in the relationship between pantoyltaurine and pantothenic acid in their effect upon rats. Pantoyltaurine prepared from (+)a-hydroxy-β-β-dimethyl-γ-butyrolactone and the sodium salt of taurine according to the procedure of Snell[1] was obtained in pure crystalline form through the courtesy of Dr. J. R. Stevens of Merck & Co., Inc.

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[1] Snell, E. E., *J. Biol. Chem.*, 1941, **141**, 121.

[2] McIlwain, H., *Biochem. J.*, 1942, **36**, 417.

[3] McIlwain, H., *Brit. J. Exp. Path.*, 1940, **21**, 136.

The experiments were conducted on young male albino rats. At 3 weeks of age the animals were placed on a diet consisting of dextrose 68%; vitamin-free casein 18%; hydrogenated vegetable oil (crisco) 8%; salt mixture U.S.P. XI No. 1 4%; cod liver oil 2%; supplemented with 0.8 mg each of thiamin, riboflavin and pyridoxine, 10 mg of nicotinamide, and 100 mg of choline chloride per 100 g of diet. Calcium pantothenate (dextrorotatory) and pantoyltaurine were fed separately to the animals by stomach tube.

The growth promoting effect of the daily administration of graded doses of calcium pantothenate, of pantoyltaurine or of a combination of both is illustrated in Fig. 1, each curve representing the average weight of 6 rats. The daily administration of 100 γ or of 1 mg pantoyltaurine (curve 2 and 3) did not influence the growth of the rats as compared with that of the control group (curve 1). The average survival time in these 3 groups was the same (21 days), and adrenal hemorrhages were found alike in these groups on gross examination at autopsy. Daily feeding of 1 mg pantoyltaurine to rats receiving 25 γ of pantothenic acid did not change the growth rate (curve 4 and 5) nor did it influence the development of signs of pantothenic acid deficiency; all rats maintained on 25 γ of pantothenic acid daily, exhibited to a comparable degree deficiency signs such as coarseness and rustiness of the fur and porphyrine deposits on their whisker. No deaths were observed in these groups over the period of 80 days. Likewise, no significant difference in weight nor in appearance was noted between rats maintained on a daily supplement of 100 γ of pantothenic acid and those receiving 100 γ of pantothenic acid plus 1 mg pantoyltaurine (curve 6 and 7). A superior weight response to a supplement of dried whole beef liver was obtained even in the presence of 1 mg of pantoyltaurine (curve 8).

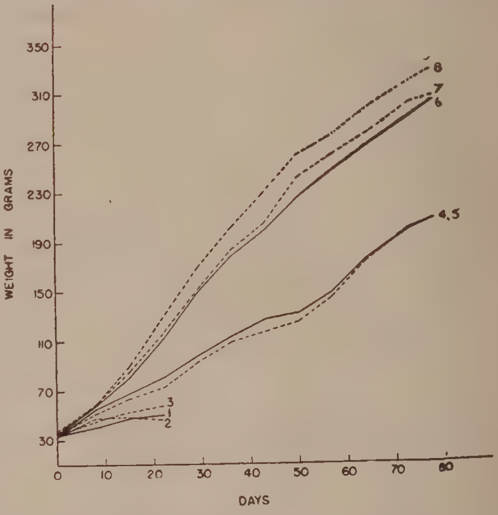

FIG. 1.

Growth response of rats maintained on a pantothenic acid deficient diet to daily feeding of graded doses of pantoyltaurine and pantothenic acid.

1. Basal diet control; 2. 100 γ pantoyltaurine; 3. 1 mg pantoyltaurine; 4. 25 γ calcium pantothenate; 5. 25 γ calcium pantothenate and 1 mg pantoyltaurine; 6. 100 γ calcium pantothenate; 7. 100 γ calcium pantothenate and 1 mg pantoyltaurine; 8. 500 mg whole dried beef liver and 1 mg pantoyltaurine.

A second experiment was carried out in which a daily dose of 25 mg of pantoyltaurine per rat (corresponding to 500 mg per kg for the weanling rats) was fed over a period of 7 weeks. This amount of pantoyltaurine likewise had no effect on growth.* In the absence of pantothenic acid it failed to enhance the deficiency symptoms or to accelerate death. When fed to rats receiving 25 γ and 100 γ of calcium pantothenate respectively, it failed to depress the weight curve or to precipitate symptoms of deficiency.

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[4] Unna, Klaus, and Mushett, C. W., *Am. J. Physiol.*, 1942, **135**, 267.

Discussion. The data presented demonstrate that feeding of pantoyltaurine to rats maintained on a diet free from pantothenic acid does not affect the growth nor the survival time. Daily administration of 1 and 25 mg of pantoyltaurine respectively to rats receiving graded doses of pantothenic acid did not influence significantly the growth of these animals. Our data, therefore, do not adduce evidence of an interference with the utilization of pantothenic acid by pantoyltaurine in animals.

While these experiments were in progress, two reports appeared on the effect of pantoyltaurine in mice. Snell *et al.*[5] reported that mice maintained on Purina Fox Chow and fed daily 200 mg per kg of pantoyltaurine showed after 3 weeks rough fur and porphyrin deposits on the whiskers. However, no attempt was made to correct these manifestations which were interpreted as symptoms of pantothenic acid deficiency, by the addition of pantothenic acid or the withdrawal of pantoyltaurine. On the other hand, Woolley and White[6] were unable to produce symptoms of pantothenic acid deficiency in weanling mice maintained on purified diets to which 1% of pantoyltaurine was added; their animals received pantoyltaurine at a level 2000 times greater than that of pantothenic acid. Likewise, hamsters fed pantoyltaurine failed to show pantothenic acid deficiency symptoms.[6] The results of our experiments on rats are in agreement with the findings of Woolley and White.[6]

Summary. Feeding of pantoyltaurine failed to produce symptoms of pantothenic acid deficiency in rats. An antagonism between the two substances similar to that established in studies on bacterial growth could not be demonstrated.

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[5] Snell, E. E., Chan, A., Spiridanoff, S., Way, E. L., and Leake, C. D., *Science*, 1943, **97**, 168.

[6] Woolley, D. W., and White, A. G. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 106.

14302

The Necessity of Additional B-Complex Factors in the Diet of the Chick.

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Factor U, a chick growth factor, may be identical with a bacterial growth factor which occurs in an eluate fraction from norite adsorbate.[1-6] The name "folic acid" has been proposed for the bacterial factor.[6]

Identification of folic acid with a chick "eluate factor" appears to have been accomplished with the isolation of the former in crystalline state, and the demonstration of high growth-promoting activity for bacteria and for chicks, as well as an anemia-prevent-

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[1] Snell, E. E., and Peterson, W. H., *J. Bact.*, 1940, **39**, 273.

[2] Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

[3] Hutchings, B. L., Bohonos, N., Hegsted, D. M., Elvehjem, C. A., and Peterson, W. H., *J. Biol. Chem.*, 1941, **140**, 681.

[4] Mills, R. C., Briggs, G. M., Jr., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 186.

[5] Stokstad, E. L. R., *J. Biol. Chem.*, 1941, **139**, 475.

[6] Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1941, **63**, 2284.

TABLE I.
Effects of Concentrates of the *Lactobacillus casei* Factor on Growth and Survival of Chicks from Hens on Experimental Diets.

					Age of chicks			
					2 wks		4 wks	
Exp.	Group	No. of chicks	Chick diet*	Hen diet*	Avg wt, g	Mortality, %	Avg wt, g	Mortality, %
I	1	10	Uns	Practical	59	30	76	70
	2	9	SupA	”	74	0	117	0
II	1	17	Uns	Uns	47	94	—	100
	2	11	SupA	”	69	10	117	10
	3	10	”	”	67	14	120	19
III	1	19	Uns	”	36	95	—	100
	2	12	”	SupC	55	8	58	75
	3	12	SupC	SupB	76	8	130	17
IV	1	15	Uns	Uns	47	93		
	2	15	”	SupB	62	7		
	3	15	”	SupC	65	7		

* Uns = unsupplemented diet, SupA = diet containing preparation A to provide 250 μg of the *L. casei* factor per 100 g, SupB = diet containing preparation B to provide 150 μg per 100 g, SupC = diet containing preparation C to provide 150 μg per 100 g.

ing (vitamin B_c) activity.[7] Recently, it has been postulated that 2 unidentified B-complex factors, different from folic acid, are also required by the chick.[8] It appears, however, that the original folic acid may be multiple in character, since preparations have been reported which are relatively more active for *Streptococcus lactis* than for *Lactobacillus casei*.[9]

It is the purpose of the present report to offer further evidence on the effects of the *Lactobacillus casei* factor on chick growth and survival, in reference to the diet of both the chick and the parent hen. S. C. White Leghorn chicks were obtained from hens fed an experimental diet to be described in another report. This hen diet is deficient in at least one factor contained in an eluate fraction and needed for normal hatchability.[10] The chicks were placed at once upon a synthetic type of diet plus supplements as indicated later.

The chick basal diet contained fibrin, 1; water and ethanol-washed casein, 20; gelatin, 4; cellulose, 5; calcium gluconate, 5; choline chloride, 0.2; inositol, 0.1; cholic acid, 0.1; salt mixture, 4.3;[11] crude soybean oil, 5; fish oil (100D-1000A), 1.0; vitamin supplements; and cerelose to complete 100 parts. Vitamin supplements as mg per kg of ration were: thiamin, 5; riboflavin, 10; pyridoxine, 5; calcium pantothenate (dl), 40; nicotinic acid, 50; 2-methyl-l, 4-naphthohydroquinone diphosphate (tetra sodium salt), 10; and a commercial biotin source, known by assay to be free of the *L. casei* factor, in an amount sufficient to prevent all indications of biotin deficiency. The preparations of the *L. casei* factor consisted of (A), a crude calcium salt; (B), a further refined preparation; (C), an eluate fraction prepared by Dr. S. Lepkovsky according to a published procedure;[1] and (D), a preparation approximately 9% pure.*

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[7] Pfiffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science*, 1943, **97**, 404.

[8] Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1943, **148**, 163.

[9] Keresztesy, J. C., Rickes, E. L., and Stokes, J. L., *Science*, 1943, **97**, 465.

[10] Cravens, W. W., Sebesta, E. E., Halpin, J. G., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 106.

[11] Almquist, H. J., and Mecchi, E., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 526.

* The writer is greatly indebted to Dr. E. L. R. Stokstad, Lederle Laboratories, Inc., for all assays and for concentrates of the *L. casei* factor A, B and D employed in this work.

TABLE II.
Effects of Supplements of the *Lactobacillus casei* Factor and Additional Unidentified Factors on Chick Growth.

Supplement and amount of the *L. casei* factor per 100 g diet μg	Supplement of additional factors per 100 g diet	No. of chicks	Avg chick wt at 2 weeks g
0	none	29	55
C, 84	”	10	73
C, 150	”	25	73
C, 250	”	10	77
D, 150	”	16	60
C, 150	SL*, 2g	15	90
”	YE†, ≅ 6g	15	92
Practical diet only	none	38	89

* Solubilized liver.
† A 50% methanol extract of yeast, concentrated to remove methanol.

The level fed in chick diets was at least 50% greater than the requirement of the chick so far as reported.[3]

The results given in Table I (Exp. I) show a distinct difference in the growth and survival of chicks from hens on a normal breeding diet, depending upon the supplementation of the chick diet. In Exp. II it was found that, when fed the basal chick diet, chicks from hens fed an unsupplemented experimental diet showed particularly severe mortality, most of them dying within 2 weeks. These chicks also showed a very poor growth, as compared to chicks of the same origin receiving a supplement of the *L. casei* factor in their diet. In Experiments III and IV the same findings in respect to extremely heavy mortality and poor growth of chicks were obtained. Similarly fed chicks which originated with hens receiving a supplement of the *L. casei* factor in their diets showed comparatively good survival to at least 2 weeks, and an intermediate rate of growth.

Such results indicate an appreciable "carryover" of a factor (or factors) from the hen diet to the chick. This carryover was essentially the same whether supplement B or C was fed the hens. After 2 weeks, however, this reserve was depleted, the chicks ceased to grow and mortality became severe. Prior to death many chicks became extremely weak, resembling chicks previously described,[12] but it is doubtful whether such chicks should be referred to as "paralyzed."

The maximal weights attained under the most favorable conditions were considerably below the normal weights for the strain and species of birds used, being approximately 120-130 g at 4 weeks, while a normal weight for chicks of this age and breed is approximately 240 g.

In comparing the growth of chicks on diets supplemented with the *L. casei* factor with the growth of chicks receiving additional supplements (Table II) it became apparent that preparations A, C and D would permit no more than a distinctly subnormal rate of growth, although mortality was largely prevented. The purest of these preparations, D, permitted the least growth. Additional crude supplements, such as solubilized liver and yeast extract, caused an increase in rate of growth to normal (approximately 90 g weight at 2 weeks). This increase may be attributed to an additional unknown factor (or factors), as previously suggested.[4,12]

Summary. Concentrates of the *Lactobacillus casei* factor in the diet of the hen affect the growth and survival of chicks fed diets deficient in this factor. In addition, at least one unknown member of the B-complex is required by chicks.

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[12] Briggs, G. M., Jr., Luckey, T. D., Mills, R. C., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 7.

14303

Comparative Effects on Experimental Tuberculosis of 2,4′-Dichlorobenzophenone and 4,4′-Diaminodiphenylsulfone.

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The bacteriostatic action *in vitro* of benzophenone and of many of its derivatives on cultures of tubercle bacilli was tested by Freedlander.[1] The most pronounced inhibitory action was exerted by 2,4′-dichlorobenzophenone. In addition to being markedly bacteriostatic for a culture of a rapidly growing avirulent strain of human tubercle bacilli, this compound was also highly effective in inhibiting the growth of cultures of a recently isolated slow growing virulent strain of the organism. The highest dilution in which bacteriostatic action was effective for the avirulent strain was 1:100,000 while for the virulent strain inhibition was recorded in a dilution of 1:60,000, the highest dilution tested. Sodium p,p′-diaminodiphenylsulfone-N,N′-didextrose sulfonate (promin) was also tested and the results were quite inferior to those obtained from most of the chlorine derivatives of benzophenone.

The high bacteriostatic capacity of 2,4′-dichlorobenzophenone against tubercle bacilli *in vitro* suggested the desirability of an experiment to determine what effect, if any, this compound might have against virulent tubercle bacilli *in vivo*.

Previous observations had indicated that one of the most effective compounds in deterring experimental tuberculosis in guinea pigs is 4,4′-diaminodiphenylsulfone.[2,3] Consequently an experiment was planned so that (1) the results obtained from 2,4′-dichlorobenzophenone could be compared with the results obtained from a drug of established tuberculotherapeutic efficacy and (2) the results following the administration of the drugs could be compared with the results in animals that were not treated.

Methods. Four groups, each consisting of 10 adult male guinea pigs, were used. The respective groups were identified as follows: Group 1, infected but not treated (controls); Group 2, infected and treated with 0.33% of 4,4′-diaminodiphenylsulfone in the feed; Group 3, infected and treated with 0.5% of 2,4′-dichlorobenzophenone in the feed, and Group 4, infected and treated with 1.0% of 2,4′-dichlorobenzophenone in the feed.* The administration of the drugs was started 2 days before the animals were inoculated with tubercle bacilli. All animals received subcutaneously in the sternal region 0.1 mg of a 20-day-old culture of the virulent variant of H37 (H37RV).† The guinea pigs were caged in pairs and the experiment was terminated 60 days after the animals had been inoculated with tubercle bacilli. At the time of necropsy tissues from the liver, spleen, lungs, tracheobronchial lymph nodes, the site of inoculation and the contiguous lymph nodes were preserved for subsequent histologic examination. In addition, approximately half of each spleen of the guinea pigs

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[1] Freedlander, B. C., Proc. Soc. Exp. Biol. and Med., 1942, **52**, 153.

[2] Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Am. Rev. Tuberc.*, 1942, **45**, 303.

[3] Feldman, W. H., Hinshaw, H. C., and Moses, H. E., unpublished data.

* Preliminary trials had indicated that guinea pigs weighing from 400 to 500 g would consume an average of 40 g of feed daily, containing 1% by weight of 2,4′-dichlorobenzophenone. When the amount of the drug was increased to 2% and to 4% of the daily food intake inappetence developed and most of the animals receiving feed containing 4% of the drug died within 3 weeks. Both compounds used in this study were supplied through the kindness of Dr. L. A. Sweet, Parke, Davis & Co., Detroit, Michigan.

† The origin and characteristics of H37RV have been described previously.[2]

TABLE I.
Summary of Comparative Effects of 4,4′diaminodiphenylsulfone and of 2,4′-dichlorobenzophenone on Experimental Tuberculosis of Guinea Pigs. All Animals Received Subcutaneously 0.1 mg of Human Tubercle Bacilli (Strain H37RV). Drugs Were Supplied Daily in the Feed. Duration of Experiment, 62 Days.

	Numerical index of infection determined microscopically				
Group	Spleen (Max. 35)	Lungs* (Max. 30)	Liver (Max. 25)	Site of inoculation* (Max. 10)	Avg index (Max. 100)
1. Controls	26.1	19	16.3	10	71.4
2. Treated†	2.9	3.8	1.2	6.4	14.3
3. Treated‡	25	18.5	21.5	10	75.0
4. Treated§	27.8	16	19	10	72.8

* Includes contiguous lymph nodes.
† 4,4′-diaminodiphenylsulfone, 0.33% of the feed.
‡ 2,4′-dichlorobenzophenone, 0.5% ” ” ”
§ 2,4′-dichlorobenzophenone, 1.0% ” ” ”

in Groups 1, 2 and 4 was removed aseptically and utilized for attempts to culture tubercle bacilli.

Results. Animals surviving. Of the 40 animals inoculated with tubercle bacilli when the experiment began, 34 were living 60 days later. All of the control or untreated group survived; of those that received 4,4′-diaminodiphenylsulfone seven were living; of those that received in the feed 0.5% of 2,4′-dichlorobenzophenone, 9 were living; while of the 10 that received in the feed 1% of the benzophenone derivative, 8 were living. The cause of death of the 3 animals in the group that received 4,4′-diaminodiphenylsulfone was not evident at necropsy. Each had residual tuberculous changes at the site where tubercle bacilli had been injected and in one animal there was a slight progressive tuberculous involvement of the spleen. In none of the 3 was there sufficient tuberculosis to account for death. The opposite was true of the 3 animals which died while receiving 2,4′-dichlorobenzophenone. In each of these there was present severe tuberculosis that appeared sufficient to have been lethal.

Relative amounts of tuberculosis. For the sake of brevity the comparative amounts of tuberculosis in the organs of predilection and at the site of inoculation of the four groups of guinea pigs have been summarized in Table I.‡

The results show that there was no significant difference in the severity of the tuberculosis in the control group and in the 2 groups of guinea pigs that received 2,4′-dichlorobenzophenone. In contradistinction to the results obtained from the chlorine derivative of benzophenone were the effects exerted by 4,4′-diaminodiphenylsulfone. Whereas the average numerical index of infection (on the basis of 100) was 71.4 for the untreated animals the average index of infection of those animals treated with the sulfone compound was only 14.3. Not only was there markedly less tuberculosis in the latter group than was true of the untreated controls and the animals that received 2,4′-dichlorobenzophenone, but of much more importance was the fact that the histologic character of the tuberculous processes in the group that received the sulfone compound was with few exceptions that of a nonprogressive or arrested process.

The results indicated definitely that under the conditions imposed, 2,4′-dichlorobenzophenone did not influence the expected course of the disease favorably. This inability on the part of 2,4′-dichlorobenzophenone to exert a deterrent effect becomes especially impressive when compared with the results obtained under the same conditions with 4,4′-diaminodiphenylsulfone.

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‡ The scheme followed in recording numerically the amount of tuberculosis in experimentally infected guinea pigs takes into account the degree of involvement and the progressive or nonprogressive character of the tuberculous changes. This scheme and the importance of the microscopic examination of tissues in tuberculochemotherapeutic studies have been described previously.[4]

[4] Feldman, W. H., *Am. Rev. Tuberc.*, 1943, **48**, 248.

Effects on the blood. The only observations made on the changes in the blood of the 4 groups of animals were limited to determinations of hemoglobin.§ When the animals were killed 60 days after infection specimens of blood were obtained by cardiac puncture from 3 of the groups. The results expressed in grams of hemoglobin per 100 cc of blood are as follows: Group 1, untreated controls, 13.3; Group 2, treated with 4,4′-diaminodiphenylsulfone, 10.2; Group 3, treated with 1% of 2,4′-dichlorobenzophenone by weight in the feed, 11.2. If one assumes that the average concentration of hemoglobin for normal guinea pigs is 14 to 15 g per 100 cc of blood it is evident that neither drug reduced the amount of hemoglobin to critical levels.

Blood concentrations of the drugs. When the experiment was terminated the concentration of 4,4′-diaminodiphenylsulfone in the animals in Group 2 ranged from 1.8 to 5.7 mg per 100 cc of blood, the average being 4.1 mg. No determinations were made concerning the concentration in the blood of 2,4′-dichlorobenzophenone. We are not aware of practical methods whereby this can be accomplished.

Results of splenic cultures. Among the 10 spleens cultured for tubercle bacilli from the group of untreated controls acid-fast bacilli were obtained from 9. Seven splenic cultures were made from the group that received 4,4′-diaminodiphenylsulfone; the results were negative for 5 and positive for 2. Eight spleens were cultured from the animals in Group 4 (1% of 2,4′-dichlorobenzophenone by weight of the feed) and acid-fast bacilli were obtained from each.

Summary and conclusions. The effect of 2,4′-dichlorobenzophenone on experimental tuberculosis of guinea pigs was studied. Although 4,4′-diaminodiphenylsulfone was capable of exerting an impressive inhibitory effect, the chlorine derivative of benzophenone did not retard or influence favorably the course or character of the disease as exemplified among the animals in the untreated or control group. The following conclusions are drawn: 1. Favorable bacteriostatic results *in vitro* of chemical compounds against tubercle bacilli are not necessarily indicative of favorable results in experiments *in vivo*. 2. 4,4′-diaminodiphenylsulfone under the conditions imposed proved capable of combating a tuberculous infection rather satisfactorily. 3. The one chlorine derivative of benzophenone studied proved ineffective in altering the expected course of the disease.

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§ The hemoglobin values were determined by the Cenco-Sheard-Sanford photelometer.

14304

Effect of Some Chemicals, Which Affect Smooth Muscle Contraction, on Ciliary Activity in *Paramecium*.

JOSEPH F. OLIPHANT. (Introduced by D. M. Whitaker.)

From the School of Biological Sciences, Stanford University.

Mast and Nadler,[1] Merton,[2] Oliphant[3] and others have shown that monovalent cation salts and hydrates induce reversal in the direction of the effective beat of the cilia in protozoa while, with few exceptions, bi- and tri-valent cation salts and hydrates do not. Similar observations have been made regarding ciliary reversal in the metazoa, and the reproductive cells of plants and animals have been observed to swim backward.

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[1] Mast, S. O., and Nadler, J. E., *J. Morph. and Physiol.*, 1926, **43**, 105.

[2] Merton, H., *Biol. Zentralbl.*, 1935, **55**, 268.

[3] Oliphant, J. F., *Physiol. Zool.*, 1942, **15**, 443.

Alverdes,[4] Worley[5] and others have also shown that in fragments of ciliated protozoa the cilia continue to beat as long as they are connected with a fragment of cytoplasm. Such ciliated fragments have also been observed to reverse direction of ciliary beat in response to environmental change and this suggests that cilia here are automatic in their activity and that alterations in ciliary behavior are of an extraneous nature. Furthermore it is now generally agreed that the fundamental phenomena of protoplasmic contractility are essentially alike for pseudopodial movement, ciliary activity and muscular contraction, and that a theory which will adequately explain one will doubtless be applicable to the others.[6–8] However the demonstration of similarity between various forms of cellular movement is by no means complete.

From these observations and assumptions it would be expected that substances which profoundly affect cellular movement in populations of smooth muscle cells would also profoundly affect ciliary activity, possibly inducing ciliary reversal. It is the object of this investigation to explore the possibility of such a similarity in behavior of ciliated and smooth muscle cells in response to chemical agents in the environment, with a view to contributing to the analysis of chemical and physical factors involved in protoplasmic contractility.

Methods. Paramecium multimicronucleatum was used because of its size and the readiness with which it lends itself to culture and experimentation. In testing the effects of chemical agents on ciliary activity paramecia were first thoroughly washed and adapted to a balanced salt solution. 2.5 cc of solution to be tested were put into a modified Columbia culture dish on the stage of a dissecting binocular microscope. One of the washed paramecia was taken up in a capillary pipette and put into the test solution. The specimen was then observed continuously until the end of the experiment and if ciliary reversal occurred its duration was measured with a stop watch. This was repeated with other specimens as often as was desired and ciliary reversal was only recorded as having occurred if the animals actually swam backward for a time readily measurable with a stop watch. No experiments were actually performed on smooth muscle cells, it being believed that the action on them of the chemicals used is sufficiently well established.

*Experimental results.** The results of an extensive series of experiments are summarized in Table I. In the first column are listed the different chemicals tested. The second column shows the range of concentrations over which the chemicals were tested. It is not practicable or particularly instructive to list each concentration investigated. In the third column is given the ciliary response to each chemical. It was observed that the behavior was not the same in all instances, even with substances inducing ciliary reversal; and the duration of the initial period of ciliary reversal, which was often followed by recovery and subsequent reversals, varied greatly in the different chemicals. It is not possible to summarize the detailed response in each solution; the third column only indicates qualitatively the nature of the ciliary response. In general, duration of ciliary reversal varied directly with the concentration of the chemical inducing it.

Discussion. The results show that those substances which induce violent contractions in smooth muscle cells regardless of innervation also induce reversal in direction of the effective beat of the cilia of *Paramecium.*

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[4] Alverdes, F., *Studien Infusorien uber Flimmerbewegung Lokomotion und Reizbeantwortung,* 1922, Berlin, 123s.

[5] Worley, L. G., *J. Cell. and Comp. Physiol.,* 1934, **5**, 53.

[6] Hartmann, M., *Allgemeine Biologie,* 1933, Jena, 792s.

[7] Gray, J., *Ciliary Movement,* 1928, Cambridge, 162 pp.

[8] Gray, J., *Experimental Cytology,* 1931, Cambridge, 516 pp.

* The author is indebted to the Department of Physiology, The Lane Hospital, and Dr. E. L. Tatum for supplying most of the chemicals used in this investigation.

TABLE I.

Effect of Increase in Concentration of Various Chemicals in the Environment on Reversal in Ciliary Action in *Paramecium.*

Substance tested	Range of concentrations*	Duration of initial ciliary reversal†	pH‡
$BaCl_2$	M/250-M/500,000	1.0-2.5 min. in optimum higher concentrations	5.5-6.5
$Ba(OH)_2$	M/300-M/500,000	1.0-3.0 min.	7.5-10.5
Physostigmine sulfate	M/250-M/1,000	20-30 sec.	5.8-6.8
Physostigmine salicylate	M/100-M/1,000	25-40 "	5.9-6.5
Triturus embryo toxin	1:50 to 1:1,000	40-60 "	6.3-7.2
Novocain (procain hydrochloride)	0.5% to 0.0001%	0.5-1.5 min.	7.2-7.5
Colchicine	M/150 to M/5,000	3.0-5.0 sec.	7.0-6.5
Papaverine	1:20,000 and lower	Reversal (Hopkins)[9]	7.8[9]
Morphine sulfate	1:500 and higher	" "	
Codeine sulfate	1:500 " "	" "	

* Atropine sulfate, choline, acetyl-choline (bromide), nicotine sulfate, histamin (dihydrochloride), histidine, strychnine sulfate and nitrate, and caffein citrate were tested in concentrations from those immediately lethal to those of such dilution as would induce no or only a very weak shock reaction and in no concentration of any of them was there ciliary reversal. In histamine there were a series of avoiding reactions in which the reversal phase was less than a second. Avoiding reactions did not occur in other solutions and while behavior was not the same in all, typically, there was a marked increase in rate of forward locomotion, with sudden changes in direction of movement, lasting several minutes and often followed by subsequent shock reactions.

† Initial reversal is often followed by recovery and subsequent reversals. Range rather than averages are indicated.

‡ H-ion concentration was sometimes adjusted by addition of sodium carbonate or other substances.

This is certainly true for the barium compounds and for physostigmine; and with the probable exception of papaverine the narcotics used by Hopkins (and novocaine also), in concentrations inducing ciliary reversal, produce violent contractions of smooth muscle regardless of innervation. The action of colchicine on smooth muscle may not be sufficiently well established to warrant comparison and only preliminary studies have been made on Triturus embryo toxin (extracted from eggs of Triturus, see Tatum *et al.*),[10] however, there are indications that they too induce contraction in denervated smooth muscle. Those substances tested which relax smooth muscle or act on smooth muscle through the nervous system do not induce ciliary reversal. Histamine may be an exception and marked avoiding reactions were observed in histamine.

These results are empirical, however they do further suggest that the chemico-physical changes involved in ciliary activity and smooth muscle contraction may be fundamentally alike. It has been suggested by Mast and Nadler[11] and Oliphant[12] that ciliary reversal is associated with, among other factors, differential absorption of cations and an increase in cytoplasmic viscosity. Hopkins,[13] without having made actual measurements, attributes the action of the narcotics to volume changes through uptake or loss of water.

These experiments further suggest that protozoa may offer more satisfactory material than populations of smooth muscle cells for the analysis of chemico-physical factors involved in protoplasmic contractility. They are large, easily handled, single cells may be studied, and it is possible to obtain any desired quantity of experimental material whose genetic and environmental conditions may be satisfactorily standardized and so critically studied.

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[9] Hopkins, H. S., *Am. J. Physiol.*, 1922, **61**, 551.

[10] Horsburgh, D. B., Tatum, E. L., and Hall, V. E., *J. Pharm. and Exp. Therap.*, 1940, **68**, 284.

[11] Mast, S. O., and Nadler, J. E., *loc.* cited.

[12] Oliphant, J. F., *loc.* cited.

[13] Hopkins, H. F., *loc.* cited.

14305

Pregnancy Maintenance in Hypophysectomized-Oöphorectomized Rats Injected with Estrone and Progesterone.*

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Pregnancy is quickly terminated in the rat if hypophysectomy is performed before day 11, but is not interrupted in the majority of cases if the operation is done after this time.[1,2] Other experiments[3,4] have made it seem likely that the pituitary is indispensable in the first half of pregnancy only because of its follicle- and interstitial-cell-stimulating hormones which in the proper ratio and dosage will cause estrogen secretion by the ovaries of an hypophysectomized rat, and the lactogenic hormone which stimulates the hypophysectomized rat's luteal tissue to secrete progesterone. Further support to this proposition has been given by the data submitted in this report.

Experimental. Rats of the Long-Evans strain, 60-70 days of age, in pro-oestrus were caged with males overnight and those showing vaginal sperm on the following morning (day 1) were hypophysectomized† and oöphorectomized (2 were also adrenalectomized) one week later (days 7-8). At the time of oöphorectomy, implantations were checked. All animals received 200 mg of glucose intraperitoneally 4 times weekly for at least one week postoperatively and were provided with a wet as well as a dry diet. Hormonal injections were begun immediately after operations and continued until the day before autopsy. Estrone in 0.1 cc of sesame oil was injected subcutaneously in a daily dose of 1 μg (10 I.U.) alone and with 2 daily levels (3 and 4 mg or I.U.) of progesterone, also given subcutaneously in 0.1 cc of sesame oil. Progesterone was also injected alone in daily doses of 3 and 4 mg. Three rats doubly-operated on day 9 received no hormonal treatment. The progressive growth or resorption of the uterine contents was checked daily by gentle palpation and records made of the nature of the vaginal smear. In a few instances where the uteri were palpably empty the rats were sacrificed before the time of normal term; otherwise necropsies were carried out from day 21 to 24. Rats 7 and 14 (see Table I) died at term after having been observed in unsuccessful labor. The other mothers were chloroformed and the young quickly taken from the uterus, stript of membranes, weighed and, if alive, usually given to a foster mother. Approximate figures for gestational span, size of litter and average birth weight for this colony are 22 days, 9 young and 5 g respectively.

Results. The results are listed in the accompanying table. Seven rats hypophysectomized and oöphorectomized on days 7 or 8 of pregnancy and injected daily with 1 μg of estrone and 4 mg of progesterone carried from 3 to 9 living young to term and, in addition, were found to have from 1 to 6 fetuses in different stages of resorption. One of the 7 rats died on day 22 after having attempted to deliver herself of 6 term-size fetuses. All of the living fetuses seemed to be normal and equalled or surpassed in weight

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* Aided by grants from the Research Board of the University of California and from the Rockefeller Foundation. The estrone and progesterone used in this work were generously supplied by Parke, Davis and Company.

[1] Pencharz, R. I., and Long, J. A., *Science*, 1931, **74**, 206.

[2] *Id., Am. J. Anat.*, 1933, **53**, 117.

[3] Cutuly, E., *Endocrinology*, 1942, **31**, 13.

[4] Lyons, Wm. R., Simpson, M. E., and Evans, H. M., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 134.

† The author is indebted to Miss F. Carter and Mrs. A. McKnight for hypophysectomizing these rats and to Prof. H. M. Evans for making available the facilities of the Institute of Experimental Biology.

TABLE I.
Showing That Pregnancy May Be Maintained in Hypophysectomized-Oöphorectomized Rats by Injections of Estrone Plus Progesterone.

	Daily hormonal dose			Body wt on day of				
Rat	Estrone µg	Progesterone mg	Day of Operation	Mating	Operation	Autopsy	Day of Autopsy	Uterine findings
1	1	4	8	165	204	246	K 23	9 L (48 g), 1 R
2	1	4	8	143	160	198	”	9 L (46 g), 1 R
3	1	4	8	159	184	230	”	5 L (34 g), 5 R
4	1	4	8	144	166	196	”	3 L (20 g), 6 R
5	1	4	7	141	164	210	22	7 L (40 g), 1 R
6	1	4	7	163	183	241	”	6 L (35 g), 2 R
7	1	4	7	153	182	225	D 22	6 RD (32 g), 2 R
8	1	3	7	160	180	214	K 21	7 L, 1 RD
9	1	3	7	180	213	260	”	8 L (35 g), 1 R
10	1	3	7	210	240	292	”	6 L (35 g), 1 RD
11	1	3	7	188	220	260	”	4 L (24 g), 2 RD
12	1	3	7	174	201	243	22	5 L (29 g), 3 R
13	1	3	7	160	191	234	”	6 L (42 g)
14	1	3	8	190	221	259	D 21	9 RD
15	1	3	7	180	212	235	K 22	2 RD, 5 R
16	1	3	7	206	233	198	23	10 R
17	1	3	7	176	189	170	”	No implants
18	0	3	7	178	187	177	”	” ”
19	0	3	7	202	234	219	”	11 R
20	0	3	7	205	228	204	”	9 R
21	0	3	7	218	241	232	”	11 R
22	0	3	8	170	182	212	”	10 R
23	0	3	8	188	212	192	”	6 R
24	0	3	8	187	209	212	”	11 R
25	0	4	8	154	179	225	22	1 L, 2 RD, 6 R
26	0	4	8	144	162	186	”	10 R
27	0	4	8	175	192	206	”	8 R
28	0	4	9	172	201	207	”	9 R
29	1	0	9	169	194	151	15	No implants
30	1	0	9	218	236	207	”	” ”
31	1	0	9	243	266	210	”	” ”
32	0	0	9	170	185	164	24	” ”
33	0	0	9	165	202	172	”	” ”
34	0	0	9	246	273	220	”	” ”

K = Killed. D = Died. L = Living fetuses.
R = Fetuses and placentæ being resorbed. RD = Recently dead fetuses.

To the results tabulated may be added those obtained from experiments repeated to confirm old observations. Eight rats hypophysectomized and 4 oöphorectomized within the 6th to 9th days of pregnancy and uninjected all resorbed 24-72 hours post-operatively. Of new interest were 2 rats, hypophysectomized, oöphorectomized and adrenalectomized on the 7th day of pregnancy and injected, daily, with 1 µg of estrone plus 3 mg of progesterone. One was sustained through day 25 and had 1 recently-dead, and 5 living fetuses and the other, gravid with 7 term-size fetuses, died on day 21. Similar results were obtained with 2 rats, hypophysectomized only on the day of insemination and injected daily with 1 µg of estrone and 2 mg of purified lactogenic hormone. One was still gravid with 1 recently dead and 4 living fetuses when sacrificed on day 25, and the other died on day 22 without being able to deliver its 7 large fetuses.

the normal new-born of this colony. Some of these have been successfully nourished by foster mothers, but have lagged behind, in size and weight, the normal rats of this colony. Approximately the same results were obtained with the 10 rats similarly operated and injected daily with 1 µg of estrone and 3 mg of progesterone. Six of these had from 4 to 8 living young with one to 3 resorbing or recently dead fetuses; one died on day 21 after having attempted to deliver 9 term-size fetuses; 2 had resorbing fetuses and one had an empty uterus when autopsied on days 22-23.

The 7 doubly-operated rats injected with 3 mg of progesterone and 4 injected with 4 mg of progesterone may be considered as one group since the results did not differ greatly. In only one of the 11 rats was pregnancy maintained until term, and that animal (No. 25) was gravid with one living, 2 recently-dead and 6 resorbing young when autopsied on day 22. With the exception of rat 18, which showed no implants at autopsy, all of the others still bore placentae in various stages of regression, and either no recognizable fetuses or autolysed vestiges. The 3 rats operated on day 9 and injected with 1 μg of estrone promptly resorbed as did those similarly operated and uninjected.

Discussion. It is not assumed that the dosage and ratio of estrone and progesterone found adequate in maintaining pregnancy in this study will eventually prove optimal, but the measure of success attained with 10 I.U. of the former and 3 and 4 I.U. of the latter has seemed sufficient to warrant reporting at this time. The results may be said to clarify the role played by the pituitary in pregnancy, and to call attention once more to the placenta as an organ probably capable of carrying on the pituitary's gonadotrophic and the ovary's hysterotrophic function, thus making doubly certain that an ample supply of estrogen and progestogen will be available. That the pituitary's main role in the first half of pregnancy is to secrete 3 hormones (FSH, ICSH and lactogenic) which cause the ovaries to form daily equivalents of 1 μg of estrone and 3-4 mg of progesterone is indicated by the finding that at least during the 7-11-day period when hypophysectomy causes resorption just as quickly as oöphorectomy, estrone-progesterone therapy has proven to be adequate substitution following the double operation. This is especially interesting in light of the emphasis recently placed on the so-called metabolic hormones of the pituitary which few workers would think of as functioning extensively through the mere production of estrogen and progestogen. Before declaring the pituitary hormones, other than the gonadotrophic triad, completely unessential in pregnancy, a search should be made for these or related substances in the placenta,[5,6] and the possibility still remains that the pituitary's contribution of even small amounts of these hormones might have been beneficial in increasing the number of living young carried to term in this experiment.

With new emphasis being given to the need of the pregnant rat for both estrogen and progestogen, the experiment of Haterius[7] wherein he removed from pregnant rats the ovaries and all fetuses but one (leaving the placentae) and maintained pregnancy, might be interpreted as indicating the secretion of both of these hormones by the rat placentae. It is possible that rat 26, of the present series, injected with 4 mg of progesterone alone was able to maintain its one living fetus until day 22 because of estrogen formed by the placentae of this survivor and its eight unsuccessful mates. In the rats injected with progesterone, that hormone alone or in combination with placental estrogen and progestogen was capable of supporting some of the fetuses—other than the sole survivor—for at least a week after operation, judging by the amount of fetal residue observed in some of the rats at autopsy. The striking autopsy finding in many of these cases was the survival of large (though abnormal) placentae with scarcely a trace of fetal tissue. It seems as though progesterone alone may have been partially successful in maintaining the uterine contribution to the gestational apparatus and this is in keeping with the fact that alone it is able to support[8,9] deciduomata in non-pregnant, oöphorectomized rats.

Summary. Levels of estrone and progesterone have been determined which, when given in combination to rats oöphorectomized and hypophysectomized on day 7 or 8 of pregnancy, will permit most of them to carry

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[6] *Id.*, *Sex and Internal Secretions*, Williams and Wilkins Co., Baltimore, 1939.

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a slightly less than average number of apparently normal young through the usual gestational period. Of 7 rats given 1 μg of estrone and 4 mg of progesterone daily, one died on day 22 following an attempt to deliver 6 large and 2 resorbing fetuses, and the other 6 when sacrificed on days 22 or 23 were found to have a total of 39 (3 to 9) living young and 16 (1 to 6) fetuses in various stages of resorption. Of the 10 rats given 1 μg of estrone and 3 mg of progesterone, one died on day 21 without being able to deliver 9 term-size fetuses, 6 when sacrificed on days 21 or 22 were found to have a total of 36 (4 to 8) living young and 8 either recently dead or in various stages of resorption, and the remaining 3 when sacrificed on days 22 or 23 were found to have resorbing implants or no visible-implantation sites. It may be concluded, therefore, that 1 μg of estrone and 4 mg of progesterone substituted for the ovaries and pituitary during the period of pregnancy between days 7 and 11, and for the ovaries alone from day 11 until term. The pituitary is only necessary during the first half of pregnancy in the rat, and then probably because of its gonadotrophic triad, FSH, ICSH and lactogenic hormone.

14306 P

Circulatory Effects from Cyclopropane Administered After Hemorrhage.*

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Clinical observations suggest that patients with recent extensive blood loss have an improvement of the circulation during cyclopropane anesthesia. With the onset of anesthesia, blood pressure and pulse pressure frequently increase and the pulse rate slows. These findings suggested laboratory observations under similar circumstances.

Method. Twenty-two experiments were performed on normal animals (18 dogs, 4 cats). No drugs other than cyclopropane, oxygen and a small amount of procaine were used. Arterial blood pressure determinations were made by cannulating a carotid or femoral artery after procaine infiltration. Hemorrhage was accomplished by direct rapid arterial bleeding until 25 to 30% of the estimated circulating blood volume had been withdrawn in an average of 5.7 minutes.

Cyclopropane was administered intermittently as a 30 to 50% mixture with oxygen, using the to and fro carbon dioxide absorption technic. Induction of anesthesia was accomplished with a face mask, and maintained by using an endotracheal airway. Depth of anesthesia was determined by the character of the respirations and the activity of the eyelid and swallowing reflexes. When respirations became shallow or ceased in deep anesthesia, they were aided by rhythmic manual pressure on the rebreathing bag to eliminate the effects of hypoxia and hypercapnia. Observations were made in the 4 planes of surgical anesthesia. Cyclopropane administration was started at an average time of 29 minutes following the completion of bleeding and was maintained for an average period of 44 minutes.

In 13 dogs, venous blood samples were taken for hematocrit and plasma specific gravity determinations at 3 points in the experiment: (a) before bleeding (b) prior to the onset of anesthesia (c) at the completion of anesthesia. Hematocrit cell volume was measured in Sanforth-Magath tubes and plasma specific gravity was determined by the falling drop method of Barbour and Hamilton. Electrocardiograms were obtained

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at intervals throughout the period of experiment for 9 dogs.

One cat and 2 dogs were similarly prepared but received no cyclopropane. They were observed for 4 hours during which time no rapid fluctuations in blood pressure or pulse were noted after the initial effects of hemorrhage. Oxygen was administered by tracheotomy or endotracheal airway to 3 cats and 4 dogs after hemorrhage. Its use had no appreciable effect upon circulation.

Results. Mean arterial blood pressures averaged 120 mm Hg before, and 64 mm Hg after hemorrhage. Pulse pressures averaged 58 mm prior to, and 8 mm following blood loss. At the outset, the pulse rate in the dogs averaged 87 and increased to 174 after bleeding. In cats, the pulse rate rose from 210 to 303. All animals survived the experiment.

During surgical anesthesia with cyclopropane, the highest mean blood pressures averaged 104 mm Hg, the highest pulse pressures averaged 44 mm, and the pulse rate fell to an average of 71 in the dogs and 157 in the cats. It was noted that blood pressures were usually highest in the range between lower first and upper third planes of surgical anesthesia. The pulse pressure was often largest in the third or fourth plane and the pulse rate decreased progressively as anesthesia became deeper.

In the 13 dogs that had blood studies, the results were not uniform. Hematocrits after hemorrhage increased by an average of 3% in 6, decreased 2.4% in 5, and was unchanged in 2 dogs. After cyclopropane hematocrit values increased 3.6% in 6, decreased 2.5% in 6, and remained unchanged in 1 animal as compared with the values following hemorrhage. When compared with initial, pre-hemorrhage readings, there was an average increase of 4.25% in 8, a decrease of 5.25% in 4, and no change in 1 dog.

Plasma specific gravities after bleeding showed a decrease of 0.0013 (0.45 g protein) in 11 animals, an increase of 0.0013 (0.45 g protein) in 1, and no change in the other. After cyclopropane 6 animals showed a decrease of 0.0015 (0.51 g) and 7 an increase of 0.0007 (0.24 g) as compared with the values following hemorrhage. When compared with initial control values, all 13 animals showed an average decrease in specific gravity of 0.0014 (0.48 g).

Nine animals in which electrocardiographic tracings were obtained showed various cardiac irregularities at some period during the experiment. Two showed an arrhythmia before cyclopropane administration. The irregularities noted were sinus arrhythmia, prolongation of conduction time, shifting pacemaker, premature ventricular contractions, auriculo-ventricular nodal rhythm, partial heart block, complete heart block, and multiple ventricular foci. Those of serious portent—partial and complete heart block, or multiple ventricular foci—were seen only in the deeper planes of anesthesia.

Discussion. These preliminary results tend to support the clinical observation that cyclopropane is a useful anesthetic agent in the presence of circulatory depression due to recent extensive blood loss. Its effect on blood pressure and pulse rate seem significant. The blood studies are brief and inconclusive, but the increase in plasma specific gravity after cyclopropane administration in 7 of 13 animals, when compared with the values following hemorrhage, is a deviation from the expected progressive decrease in specific gravities. All cardiac irregularities noted have been repeatedly observed since the first use of cyclopropane.[1] No animal of this group developed fatal arrhythmia. The electrocardiographic interpretations are suggestive of a vagal type of stimulation.

The mechanism of these effects from cyclopropane is not obvious. This drug exerts many parasympathomimetic effects,[2,3] increases cardiac output,[4] influences the various body fluid compartments, the spleen and other reservoirs of formed blood elements.[5]

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[3] Adriani, J., and Rovenstine, E. A., *Am. J. Physiol.*, 1941, **133**, 192.

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Conclusion. Preliminary observations seem to substantiate the clinical impression that cyclopropane is a useful anesthetic agent in the presence of circulatory depression due to rapid hemorrhage.

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14307

Yolk Sac Complement Fixation Antigen for Use in Psittacosis-Lymphogranuloma Venereum Group of Diseases.

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The recognition in recent years of the frequency of human infections with several members of the psittacosis-lymphogranuloma venereum group of viruses[1-4] has led to an increased interest in procedures which aid in the laboratory diagnosis of these diseases. The demonstration of complement-fixing antibodies in the sera of patients convalescent from psittacosis and from lymphogranuloma[7,8] has been used for establishing the etiology of these maladies. Considerable confusion has developed recently, however, regarding the interpretation of complement fixation reactions obtained in these diseases because of cross reactions displayed by the patients' sera with antigens prepared from tissues infected with the viruses of classical psittacosis and lymphogranuloma venereum and other members of this group of agents.[2,9-11] Although at present it is impossible to differentiate sharply between human psittacosis and lymphogranuloma by means of serological tests, it is possible, under appropriate conditions, to establish that a given acute illness was caused by a member of this group of viruses.[3]

The observations of several groups of workers suggest that the titers of sera from patients with psittacosis, closely related infections and with lymphogranuloma may differ somewhat when tested against homologous and heterologous antigens prepared from this group of viruses.[2,9,10] Furthermore, pigeons recovered from ornithosis develop antibodies that fix complement with meningopneumonitis antigen but not with lymphogranuloma antigen.[11] The observations reported at this time extend those of others regarding the reactions of human convalescent sera with complement-fixing antigens of psittacosis and lymphogranuloma. In addition, a comparatively simple and safe method for preparing psittacosis antigen from highly infectious material is described.

Materials and Methods. Preparation of Psittacosis Antigen. Antigens containing

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[5] Bedson, S. P., *Lancet*, 1935, **2**, 1277.

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[8] McKee, C. M., Rake, G., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 410.

[9] Rake, G., Eaton, M. D., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 528.

[10] Eaton, M. D., Martin, W. P., and Beck, M. D., *J. Exp. Med.*, 1942, **75**, 21.

[11] Eddie, B., and Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 291.

washed inactive elementary bodies of psittacosis prepared from infected agar slant cultures according to the method of Meyer and Yanamura[17] have proved highly satisfactory in our earlier diagnostic studies.[3] However, this method is not well adapted for the production of appreciable quantities of antigen mainly because infectious material is repeatedly handled during the course of differential centrifugation. A safer procedure was therefore adopted, which consisted of a number of steps previously employed by others in the preparation of virus materials. These include the use of yolk sac tissue as starting material,[4,12] inactivation of virus with formalin,[5,12] extractions with ethyl ether[12–14] and washing the virus by differential centrifugation.[3,15] A description of the method now in use follows.

The P-4 strain of psittacosis virus isolated from a dead pigeon in 1942[16] was adapted to growth in the yolk sac of embryonated hen's eggs by 20 serial passages in this organ. The yolk sacs of 30 to 90 six-day-old embryos were injected with 0.25 cc of a 1/30 dilution of sac tissue infected with adapted virus from the 20th to 30th serial passage and incubated at 37.5°C. Yolk sacs were harvested when the embryos became sluggish, usually 56 to 72 hours after injection.

After microscopic examination of an adequate sample of the sacs had shown that impression smears stained by Machievello's method were uniformly rich in elementary bodies, the entire group of yolk sacs was placed in a 500 cc bottle containing glass beads and vigorously shaken on a machine for one-half hour. Seven cc of diluent were then added for each infected sac, the mixture was agitated by hand and then stored at 5°C for 3 days. The diluent consisted of a 1/50 dilution of McIlvaine's citric-phosphate buffer solution, pH 7.6, to which 0.3% formaldehyde, U.S.P. was added. The non-infectious, crude, aqueous suspension was removed by means of a separatory funnel from the floating fat and sedimented material that had separated during storage in the cold. It was then centrifuged in 50 cc tubes in an International horizontal machine at 2,000 rpm for 15 minutes. The aqueous mid-zone was distributed in capped Lusteroid tubes with an internal diameter of 11 mm and spun in the cold in an International angle centrifuge for 2 hours at 4500 rpm. Fat and supernatant fluid were discarded and the sediment was carefully resuspended in sufficient dilute buffered saline to equal the original volume. The buffered saline was made by adding 4 volumes of 1:50 dilution of pH 7.6 buffer to 1 volume of physiological saline solution. The resuspended sediment was next thoroughly shaken in a separatory funnel with an equal volume of ethyl ether and then allowed to stand for one-half hour at room temperature. The heavy opaque emulsion of egg material separated into a moderately opalescent aqueous phase, a mid-zone of emulsion and a clear, faintly yellow layer of ether. If such a separation did not occur, 10 to 20 cc of physiological saline solution were added and the material was again shaken and allowed to stand. The aqueous suspension was removed and extracted twice more with one-half volume of ethyl ether. If the emulsion layer in the first or second extraction was excessive due to incomplete separation, this portion was removed and centrifuged in the horizontal machine at low speed for 5-10 minutes and the aqueous phase then quickly separated.

Elementary bodies were sedimented from the ether-extracted aqueous suspension by centrifugation in Lusteroid tubes in the angle machine at 4500 rpm for 2 hours. The supernatant fluid was removed from the small pellet of grayish white sediment which was resuspended in dilute buffer solution to one-half the original volume. The virus particles were again thrown down by centrifuga-

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[15] Yanamura, H. Y., and Meyer, K. F., *J. Infect. Dis.*, 1941, **68**, 1.

[16] Smadel, J. E., Wall, M. J., and Gregg, A., in press.

[17] Meyer, K. F., and Yanamura, H. Y., personal communication.

tion in the angle head and finally resuspended in an amount of buffered physiological saline solution equal to 3 cc per yolk sac. Aggregated material was removed by centrifugation in 50 cc tubes in the International horizontal machine at 2,000 rpm for 10 minutes. Microscopic examination of materials was made at various stages in the process of purification of the virus suspensions; for this purpose slides with air dried films of the suspensions were stained with 0.25% basic fuchsin. A progressive decrease in extraneous material was noted and the final antigen was found to consist almost entirely of mono-dispersed elementary body-like structures.

From the results of preliminary experiments it appears that the application of identical methods will yield satisfactory antigens from yolk sacs infected with the virus of lymphogranuloma venereum. A recently isolated strain of virus, LA,[18] partially adapted to growth in yolk sac tissue was used in this portion of the work. Sacs were taken when the embryos became sluggish, *i.e.*, 7 to 9 days after inoculation; only those found rich in elementary bodies on microscopic examination were pooled for antigen, which was tested with convalescent antisera to determine its complement binding power.

Complement Fixation Tests. Complement fixation tests were done by the technic previously described[3] in which two exact units of complement titered in the presence of psittacosis antigen were used. Complement titration was carried out with varying quantities, *i.e.*, 0.08 cc to 0.26 cc at intervals of 0.02 cc, of a 1/30 dilution of commercial lyophilized guinea pig serum. In each test a known immune serum was titered to end point with 2 units of previously standardized antigen. This titration served as a final control on the activity of the hemolytic system and permitted a comparison of the data from one experiment with that of the next.

In addition, Lygranum C. F. *Control Lot 87884 received from Dr. Clara Nigg of the Squibb Institute was used in comparative titrations studies on certain sera. This antigen, in the dilution recommended on the label, was employed with the complement system used with our antigens.

Antisera. Three groups of convalescent sera were studied for their reactions with psittacosis and lymphogranuloma venereum antigens. One group of lyophilized sera was from patients with psittacosis,[3] another group of sera, stored in the frozen state, came from cases of lymphogranuloma among Army personnel, while the final group consisted of samples of lyophilized pigeon sera obtained from birds during an epizoötic of ornithosis.[16]

Results. Antigens consisting of suspensions of washed elementary bodies of psittacosis prepared in the manner described, fixed complement in dilutions 1:4 to 1:8 when tested with appropriate dilutions of sera of patients convalescent from psittacosis. These antigens were not anticomplementary and did not react with sera from normal persons. In addition, these antigens were tested against a series of sera known to give positive Wassermann reactions as well as another series from cases clinically diagnosed as atypical pneumonia. The results of these tests were likewise negative. The physical appearance of the preparations changed little over a period of several months and the suspensions failed to become anticomplementary. However, slight diminution in specific complement binding power occurred during the 2nd and 3rd months of storage in the refrigerator; certain loss of activity has also been noted with antigen prepared from agar slant cultures. Thus, for diagnostic work it was sometimes necessary to increase the concentrations of the stored preparations in order to obtain 2 units of antigen, *e.g.*, from 1:8 to 1:6 or 1:4 to 1:3.

The fact that antigens prepared from psittacosis virus and closely related agents will fix complement with lymphogranuloma antiserum is well established.[2,9,10] We were interested in determining whether differences could be detected in a given antigen when it was titered with psittacosis and lymphogranuloma antisera, each of which was diluted so that it contained 4 units of antibody on the basis of a previous standardization with psittacosis antigen and Lygranum C.F., respectively. The results summarized in Table I indicate that antigens prepared from eggs infected with the No. 4 pigeon strain of psittacosis virus fix complement to the same titer when tested with comparable amounts of antibody from patients with psittacosis and lymphogranuloma. Similar studies with one lot of elementary bodies of lymphogranuloma suggest that slightly higher titration end points may result when homologous antiserum is used with this type of antigen (Table I).

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[18] Zarafonetis, C. J. D., in press.

* E. R. Squibb & Sons.

TABLE I.
Cross Reaction of Psittacosis and Lymphogranuloma Antigens with Convalescent Human Sera.

		Dilution of antigen			
Antigen*	Sera†	1:1	1:2	1:4	1:8
Psitt No. 5	Psitt	4	4	4	2
	L-V	4	4	4	2
	Normal	0	0	0	0
Psitt No. 6	Psitt	4	4	4	2
	L-V	4	4	4	1
	Normal	0	0	0	0
L-V No. 7	Psitt	4	4	0	0
	L-V	4	4	4	1
	Normal	0	0	0	0

* Antigens were prepared by the method described in the text.

†Four units of antibody from the same samples of convalescent human psittacosis and lymphogranuloma serum were used throughout.

4 indicates complete fixation of complement.

2 indicates 50% fixation of complement.

TABLE II.
Titration of Human and Pigeon Convalescent Sera with Psittacosis Antigen and Lygranum C.F.

		Antigen complement fixation titer*	
Name	Disease	Psittacosis	Lygranum C.F.
E.G.	Psitt	1:40	0
F.S.	”	1:80	1:40
Me.	”	1:160	1:80
F.C.	”	1:320	1:40
W.W.	L-V	1:80	1:40
L.A.	”	1:640	1:160
J.B.	”	1:40	1:40
E.M.	”	1:40	1:40
Do.	”	1:320	1:320
N.G.	”	1:640	1:640
Fr.	”	1:80	1:160
Pigeon 24	Psitt.	1:80	0
” 38	”	1:40	0
” 40	”	1:40	0

* Titers indicate the last tube demonstrating at least a 3-plus fixation.

0 Indicates no fixation with a 1/10 dilution of serum.

Series of serum specimens from patients with psittacosis and with lymphogranuloma venereum and from pigeons with psittacosis were titrated simultaneously with psittacosis antigen and Lygranum C.F. The results of these titrations are summarized in Table II. The data indicate that the end points obtained with sera from patients with psittacosis were regularly slightly higher when tested with homologous than heterologous antigen. The difference was more striking with pigeon serum than with human serum, in that the former failed entirely to react with Lygranum C.F. This agrees with meningopneumonitis studies of Eddie and Francis[11] with pigeon serum. Four of the 7 lymphogranuloma sera showed the same titer when tested with each antigen. Two of the 7 samples gave a slightly higher end-point with psittacosis antigen; while one fixed in a higher dilution with Lygranum C.F. A number of human and pigeon sera listed in Table II were titrated at other times with lymphogranuloma antigen No. 7 (Table I), the end-points for individual sera were comparable to those obtained with Lygranum C.F. antigen.

Discussion. The yolk sac complement fixation antigen is a cleaner and generally more satisfactory antigen than is the tissue agar slant preparation. The method of preparation of this antigen is simpler and a great deal safer. The relative number of elementary bodies in the preparation and its antigenicity compare favorably with the preparations made from tissue agar slants.

The serological data reported at this time indicate that the complement-fixing antigen which is common in the viruses of the psittacosis-lymphogranuloma venereum[1,2,9–11] group is present in about equal amounts in our strains of psittacosis and lymphogranuloma venereum. Not only do antigens prepared from each agent react equally with equivalent amounts of antibody from the two diseases, but also comparable amounts of antibody are generally demonstrable in patients' sera when tested with either antigen. While slight differences were found in the antibody titers of certain sera when tested with the two antigens, these were neither sufficiently great nor consistent enough to enable one to differentiate between human psittacosis and lymphogranuloma venereum by this method.

The authors wish to express their appreciation to Colonel Harry Plotz, MC, AUS, for initiating this study and for valuable suggestions rendered during the course of the investigation.

14308

Influence of Lowered Barometric Pressure on the Electroencephalogram.

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Although it is well established that a lowering of the barometric pressure accompanied by a reduction in oxygen tension produces profound changes in the electroencephalogram (EEG), little is known about the effects of reduction in barometric pressure *per se.* In general, it has been found that sensory functions which are very sensitive to anoxia are not altered by reduction in barometric pressure when the oxygen tension is kept at normal levels (*cf.* the review by Gellhorn and Hailman).[1] On the other hand, it has been reported that the cerebro-spinal fluid pressure may increase considerably even at relatively low altitudes. Armstrong[2] thinks that this increase in cerebro-spinal pressure is due to a liberation of nitrogen in gas form. Bergeret and Giordan[3] showed that a rise in cerebro-spinal pressure occurs only at such altitudes in which the oxygen tension is considerably below normal. In view of the fact that alterations in cerebro-spinal fluid pressure may affect the EEG (Forster and Nims)[4] a study of the effect of lowered barometric pressure on the EEG seems to be of interest.

The experiments were performed on 20 unanesthetized male rats which were kept singly in an atmosphere of 100% oxygen. The pressure was reduced in one minute to a level of 130 mm Hg and then maintained for 5 minutes; then the normal atmospheric conditions were restored. In a second control series, the rats were subjected to the same pressure changes in air. The EEG was recorded as in previous investigations (Gellhorn and Kessler).[5]

Results. Uniform results were obtained in 6 animals showing that even this extremely rapid lowering of the barometric pressure to 130 mm Hg did not cause any significant changes in the EEG of rats (*cf.* Fig. 1) nor was there any distinct change in behavior. If, however, the pressure was lowered to the same barometric level in the presence of air (6 experiments), the brain waves were completely abolished (Fig. 2). The experiments indicate that at least in rats the changes in EEG observed under conditions of lowered barometric pressure are solely due to a lowering of the oxygen tension. This conclusion is further supported by the evidence that if the barometric pressure is lowered below 130 mm Hg in an atmosphere of oxygen changes in behavior and EEG occur which are typical of anoxia. Results as illustrated in Fig. 3

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* Aided by grant from the Josiah Macy, Jr., Foundation.

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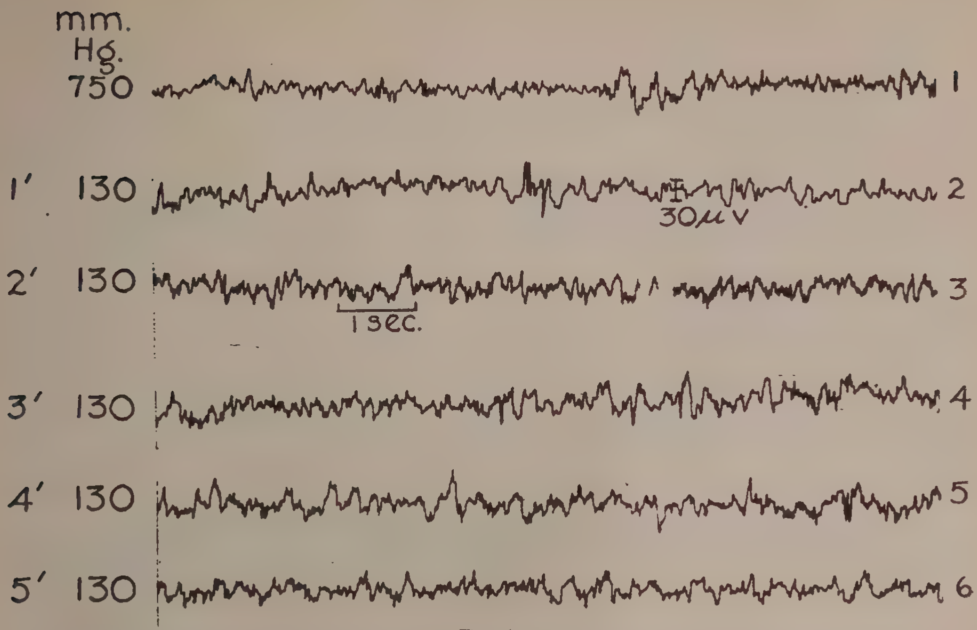

Fig. 1.

The effect of lowering barometric pressure to 130 mm Hg on the EEG of the rat in the presence of 100% oxygen. The pressure was reduced to 130 mm Hg in one minute and the rat was kept at this level for 5 minutes.

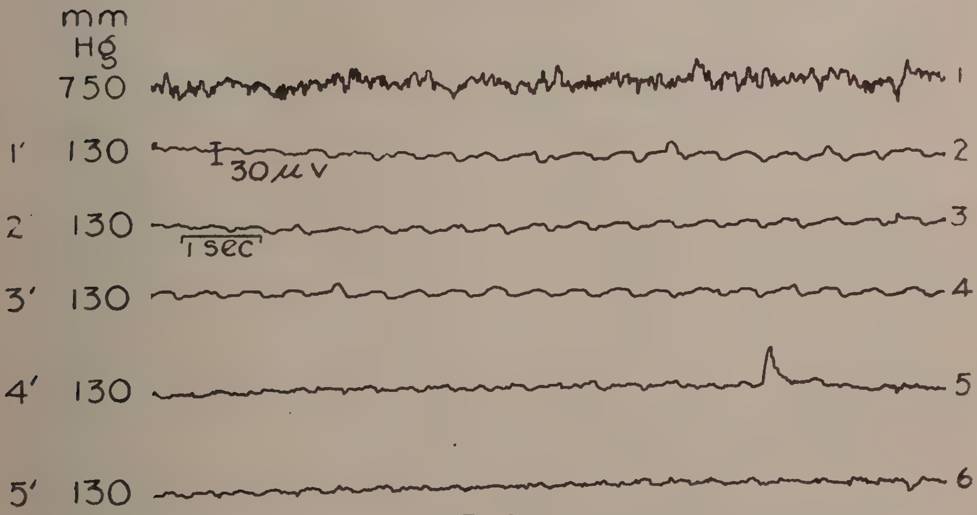

Fig. 2.

The effect of lowering barometric pressure to 130 mm Hg in the presence of air.

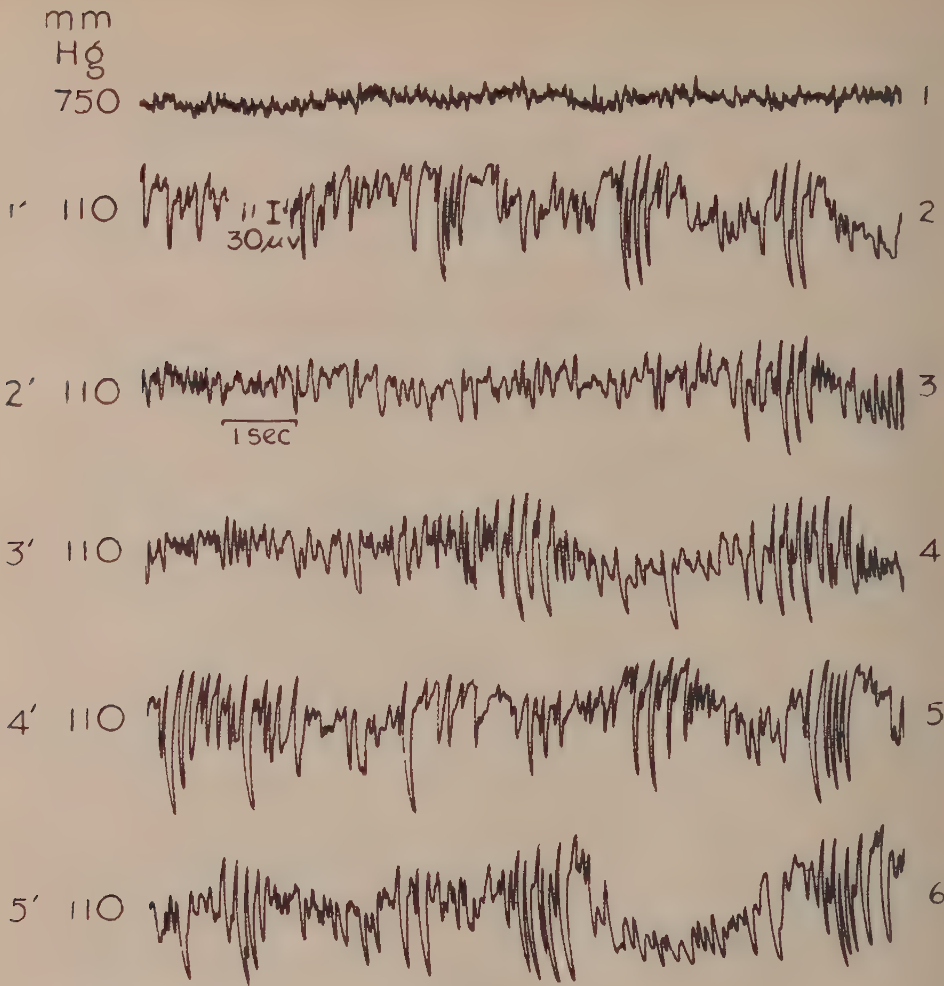

FIG. 3.
The effect of reducing the barometric pressure to 110 mm Hg in the presence of 100% oxygen.

were obtained in 8 animals. At a pressure of 110 mm (in the presence of 100% oxygen) very large potentials of a frequency of 6 to 12 per second occurred in groups (spindles). They are identical with spindles observed in rats which were exposed to similar reductions in oxygen tension by a lesser lowering of the barometric pressure in the presence of air instead of 100% oxygen.[6] In both conditions the occurrence of spindles is accompanied by a general depression of the animal (absence of spontaneous movements).

Summary. Experiments on unanesthetized rats show that rapid lowering of the barometric pressure to 130 mm Hg in 100% oxygen is not accompanied by any changes in the EEG although cortical potentials are abolished at this level in air. Further reduction in the barometric pressure leads in the presence of 100% oxygen to anoxic changes similar to those observed in the low pressure chamber in air at lesser altitudes.

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[6] Unpublished observations.

14309

Methyl Cellulose as a Colloid Laxative.

M. L. Tainter.

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Because of the shortage of vegetable gums, materials which can be more readily obtained are needed to replace them. One of the most interesting new products is methyl cellulose, which is manufactured by forming methyl ethers from purified cellulose with caustic soda and methyl chloride. This is a white product resembling cotton, which dissolves readily in cold water, forming stable colloidal solutions, which are neutral, not hydrolyzed by acids or alkali, and not subject to growth of molds. The viscosity of the solution depends on the degree of degradation of the cellulose molecule, and products are available commercially having viscosities ranging between 15 and 4000 centipoises in 2% solution.* The material flocculates out of aqueous solution when heated, but readily redissolves as it cools. In fact, a quick way to make a solution is to boil with water, and then cool in a refrigerator.

Thus far adequate pharmacologic studies of this compound have not been published. Kehoe found[1] that injection or ingestion of large amounts in white rats for as long as 6 months caused no detectable pathological changes. Hueper injected large volumes of methyl cellulose solutions in dogs and rabbits, producing effects resembling those of acacia and related colloidal materials which cannot be metabolized by the cells.[2] He also reported on its use intravenously in experimental shock.[3] Clemens and Read[4] have described its use as an emulsifier or binder in pharmaceutical and cosmetic preparations, and it is believed to be fairly widely used for such purposes at present in this country. The present observations deal with its suitability for use as a colloidal laxative similar to gum karaya and psyllium seed products.[5]

Five grams of methyl cellulose swell in a 500 cc graduate of distilled water to about 200 cc, the final volume depending on the fineness of the powder, thoroughness of wetting, etc. If complete wetting occurs, the compound disperses completely giving a thick solution or gel, the viscosity of which depends on the concentration and grade of the methyl cellulose. Swelling is not materially modified by concentrations of hydrochloric acid, sodium bicarbonate, or sodium chloride, such as it might come in contact with in the body.

Effects on Growth of Rats. To test the possible toxic effects of prolonged ingestion of the compound, 10 male and 10 female rats were used. The animals were fed Tioga meal, to which was added 10% methyl cellulose (Methocel (Dow) 4000 c.p.s.) to the food of one-half of them. In Table I are summarized the results of observations for 95 days. The male rats on methyl cellulose gained the same as the controls, and consumed the same average weight of food. In as much as the energy content of the methyl cellulose containing food was 10% less than that of the control, there must have been unrecognized inaccuracies in weighing the food eaten, possibly through scattering, or changes in the energy levels of the animals, to account for the discrepancy between food intake and growth.

The female rats on methyl cellulose gained an average of 107.3 g as compared to 125.0 g

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* Methocel (Dow Chemical Company) and Methyl Cellulose (Hercules Powder Company).

[1] Kehoe, R. A., personal communication.

[2] Hueper, W. C., *Arch. Path.*, 1942, **33**, 1.

[3] Hueper, W. C., Martin, G. J., and Thompson, M. R., *Am. J. Surg.*, 1942, **56**, 629.

[4] Clemens, H., and Reed, H. W., *Australasian J. Pharm.*, 1939, **20**, 191 (Abstracted in *J. Am. Pharm. Assn., Pharm. Abst. Section*, 1940, **6**, 25).

[5] Gray, H., and Tainter, M. L., *Am. J. Digest. Diseases*, 1941, **8**, 130.

TABLE I.

Effects of Ingestion of Methyl Cellulose (10%) in the Food for 95 Days on 20 White Rats.

Group	Avg body wt, g		Avg food intake, g a day	Avg organ wt as % of body wt				
	Initial	Final		Heart	Liver	Spleen	Kidney	Stomach
Male Controls	49.6	228.5	15.0	0.42	5.04	0.33	1.08	0.60
Male—Methyl Cellulose	51.0	230.4	15.1	0.41	4.74	0.42	1.06	0.67
Female Controls	45.6	170.6	13.9	0.40	5.00	0.49	1.11	0.76
Female—Methyl Cellulose	45.0	152.3	12.2	0.41	5.10	0.41	1.07	0.89

TABLE II.

Effects of Various Preparations of Methyl Cellulose on the Stools of Human Subjects.

Preparation	Total No. of days	Avg No. of stools a day	Avg wt stools, g a day		Avg water content of stools, %
			Wet	Dry	
Control Days	80	1.09	127.4	23.9	81.2
35% methyl cellulose + 65% bran, 5 g a day	5	1.8	145.2	31.4	78.4
The same, 10 g a day	13	1.5	160.5	30.8	80.8
3.5% MgO + 35% methyl cellulose + 61.5% bran, 5 g twice a day	11	1.5	179.5	34.5	80.7
10% MgO + 45% methyl cellulose + 45% bran, 5 g twice a day	16	1.7	177.1	29.8	83.2
10% MgO + 90% methyl cellulose, 5 g twice a day	33	1.6	230.9	35.9	84.5
Methyl cellulose, 5 g twice a day, taken as a jelly	8	1.6	232.1	40.8	82.4

for their controls. This difference seems too large to be due to accidental variation. It probably resulted from a lower food intake, since the medicated females ate only 12.2 g a day, which contained 11.0 g of the Tioga meal, as compared to 13.9 g for the controls. On a percentage basis the medicated females ate 79.9% of the food, but gained 85.8% as much weight as the controls. Hence, it appears that the medicated rats gained more than would be predicted from their food intake.

At the end of 95 days of feeding, the rats were sacrificed, autopsied and the tissues examined microscopically.† In Table I it can be seen that there was no demonstrable change in the weights of the heart, liver, spleen and kidneys. The stomachs of the methyl cellulose animals were 15% heavier than the controls, an observation made previously for other bulky diets in this department and elsewhere.[6,7] There were no significant microscopic changes which would indicate any toxic effect of the methyl cellulose on the visceral organs examined.

Laxative Effects in Man. Methyl cellulose 4000 c.p.s. was taken in various combinations with magnesium oxide powder and finely milled bran, and also alone by 3 subjects in the form of fine flakes. All subjects maintained a constant diet and collected their stools quantitatively. These were weighed before and after drying to a constant weight at 110°C. One to 2 weeks as control periods were alternated with medication periods of the same length. The combined summarized results are given in Table II.

The average wet weight of stools on the control days was 127.4 grams with approximately one evacuation a day. When methyl cellulose was mixed with various proportions of a finely milled bran, or with 3.5 or 10% of magnesium oxide, the number and volume of the stools were moderately increased. However, the results indicated that the amount of methyl cellulose administered was more important than the other materials in the quantities used. Methyl cellulose alone in a dose of 5 g twice a day increased the number of stools to 1.6 a day and the wet weight to an average of 232.1 g a day. This represented a stool volume almost double the normal. The methyl cellulose stools had a slightly higher water content than the controls, and were semi-soft and easy of passage. No colicy or griping pains were experienced as a result of the drug administration.

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† The microscopic studies were performed by Dr. A. J. Cox, Jr., professor of pathology, Stanford University School of Medicine.

[6] Addis, T., *Am. J. Physiol.*, 1931, **99**, 417.

[7] Tainter, M. L., Kulchar, G., and Stockton, A. B., *J. Am. Pharm. Assn., Scientific Edition*, 1942, **29**, 306.

The averages indicated that each gram of methyl cellulose ingested increased the bulk of the stools about 10 g. The greater part of this increase was water held by the colloid, although the dry weight increased somewhat more than could be accounted for by the dry weight of the methyl cellulose administered. Comparison of these results with those previously reported with gum karaya and psyllium[5] showed that gram for gram methyl cellulose had greater effect than these others. It would, therefore, seem to merit more extensive laboratory and clinical investigations.

The observations here reported are not enough in themselves to establish the clinical usefulness of this material. The toxicity studies carried on were not extensive enough to establish its safeness for repeated human consumption. For instance, the possibility of splitting off of the toxic methoxy groups in the body should be considered. It should be tested clinically in a variety of dysfunctions of the gastrointestinal tract to explore its general usefulness. Since it is not possible to continue these studies, the data obtained are presented in their present form for the value they may have to others interested in this problem.

Conclusions. 1. Methyl cellulose is a synthetic material having physical and chemical properties which would make it suitable for use as a colloid laxative. 2. Feeding it in a 10% concentration in the diet of rats, so they ingested an average of 11.4 g per kilo daily for 95 days, caused no change in the growth rate of the males, but decreased the growth of females about 14%, apparently as a result of decreased food intake. There were no significant pathological changes in the medicated animals observed at autopsy. 3. In doses of 10 g a day methyl cellulose approximately doubled the volume of the stools and increased their frequency in normal human subjects, a desirable clinical result sought by the use of colloidal laxatives. Each gram of methyl cellulose ingested increased the stools approximately 10 g. 4. Both the toxicological and clinical studies should be extended before a general therapeutic use of the drug as a laxative is approved.

14310

Action of Antibiotic Substances Upon *Ceratostomella ulmi*.*

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The ability of certain antibiotic agents to exert selective fungistatic and even fungicidal properties has been definitely established. This is true particularly of gliotoxin,[1] actinomycin[2] and hemipyocyanin.[3] The purpose of this investigation was to determine the effect of various antibiotic substances upon *Ceratostomella ulmi*, the causative agent of the Dutch elm disease. This problem represents two significant aspects: (a) the economic importance of this organism, and (b) its peculiar physiology, namely, its inability to synthesize vitamin B_6 or pyridoxine.[4]

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* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Soil Microbiology.

[1] Weindling, R., and Emerson, O. H., *Phytopath.*, 1936, **26**, 1068; 1937, **27**, 1175.

[2] Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1941, **42**, 231.

[3] Stokes, J. L., Peck, R. L., and Woodward, C. R., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 126.

The results of a study of the comparative effects of 7 typical antibiotic agents upon *C. ulmi* are illustrated in Table I. Two of the preparations proved to be highly effective, 2 were fairly active, one had only limited action, and 2 had no fungistatic activity at all, at least in the concentrations used. The 3 most active preparations were also strongly fungicidal.

TABLE I.
Fungistatic and Fungicidal Action of Antibiotic Substances upon *Ceratostomella ulmi.*

Nature of Substance	Mg of substance per 6 ml of nutrient broth		
	Fungistatic action		Fungicidal action in 48 hr
	Complete	Partial	
Penicillin	0	0	0
Actinomycin	0.1	0.03	0.1
Streptothricin	0	0	0
Clavacin	0.15	0.045	<0.15
Fumigacin	0	5.0	0
Hemipyocyanin	0.5	0.1	0.1
Gliotoxin	0.5	0.1	—

An attempt was made to throw some light upon the mechanism of the action of these substances upon the fungus. Robbins' medium in a slightly modified form† was used for the growth of the organism. Pyridoxine (100 μ mg per liter), peptone (5 g per liter) and malt (2.5 g per liter) were added as sources of vitamin B_6. Twenty-five ml portions of the medium were distributed in 125 ml flasks, sterilized and inoculated with the same volume of a uniform suspension of the organism grown on agar slants or in the above synthetic medium. Incubation took place at 28°-30°C for 4-7 days. Inhibition of growth was tested either by streaking agar plates or by diluting the culture and plating, using suitable agar media; the plates were examined and colonies counted after 2-4 days incubation. For measuring the total mass of growth, the combined cultures of duplicate flasks (total 50 ml) were filtered through Gooch crucibles, dried and weighed. The effect of two of the antibiotic substances found to be most effective in eliminating the growth of *C. ulmi*, namely, clavacin and actinomycin, is brought out in Table II. The nature of the medium greatly influences the extent of inhibition of the growth of the fungus by the antibiotic substances. The presence of peptone in the medium brought about reduced activity of the antibiotic substances upon the fungus. Whether this is due to the neutralization of the injurious action of the substance against a specific nutrient or function of the organism or whether the peptone served as an added nutrient remains to be determined.

In order to throw further light upon the effects of added nutrients in neutralizing the fungistatic activity of the two antibiotic substances, varying concentrations of pyridoxine and peptone were added to the above standard medium. Here as well, increasing amounts of pyridoxine or peptone were found (Table III) to neutralize partly the antibiotic effect of the two substances. The neutralizing effect was especially significant upon their fungicidal action. This was brought out in two different experiments. In one, a heavy suspension of *C. ulmi* cells was added to 25 cc portions of sterile water (Table IV) and in the other 3-day-old cultures of *C. ulmi* grown in the pyridoxine medium (Table V) were treated with the two substances. The viability of the *Ceratostomella* cells was determined by the streak method, and the actual numbers of living cells were determined by the regular plating procedure.

Actinomycin proved to possess lower fungicidal properties, as compared with its fungistatic action; clavacin is more highly fungicidal than actinomycin as compared with their respective fungistatic activities. In this respect, the fungicidal action of the two preparations is similar to their bactericidal action.[5] The presence of pyridoxine or pep-

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[4] Robbins, W. J., and Ma, R., *Bull. Torrey Bot. Club*, 1942, **69**, 184.

† 50 glucose, 2 asparagine, 1.5 KH_2PO_4, 0.5 mg $SO_4 \cdot 7H_2O$, 0.02 $CuSO_4 \cdot 5H_2O$, 0.1 $FeSO_4 \cdot 7H_2O$ and 0.09 $ZnSO_4 \cdot 7H_2O$ in 1000 distilled water.

[5] Waksman, S. A., Horning, E. S., and Spencer, E. L., *J. Bact.*, 1943, **45**, 233; Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 609.

TABLE II.

Fungistatic Action of Clavacin and Actinomycin in Presence of Different Sources of B_6*

Source of B_6 in medium	Pyridoxine		Peptone		Malt	
Antibiotic agent, mg/ml	Viability†	Fungus growth,‡ mg	Viability	Fungus growth, mg	Viability	Fungus growth, mg
None	+++	23	+++	74	+++	101
Clavacin, 0.15	+++	16	+++	73	+++	110
,, 0.6	+++	—	+++	68	+++	94
,, 1.5	+	0	++	48	+	28
Actinomycin, 0.1	0	0	+	35	0	0

* Pyridoxine 100 μ mg per liter, peptone 5 g, and malt 2.5 g per liter.

† By streak method.

‡ Weight of dry fungus mycelium in 50 ml of medium, after 7 days incubation.

TABLE III.

Influence of Pyridoxine and Peptone upon the Fungistatic Activity of Antibiotic Substances. Original medium contained 100 μ mg pyridoxine per liter. Incubation 5 days.

Clavacin			Actinomycin		
Antibiotic substance mg in 25 ml	Pyridoxine, mg	Fungus growth†	Antibiotic substance mg in 25 ml	Pyridoxine, mg	Fungus growth
0	2.5	39	0	2.5	39
1.5	2.5	12	0.1	2.5	11
1.5	5.0	7	0.1	5.0	11
3.0	2.5	0	0.1	7.5	21
3.0	5.0	8			
3.0	7.5	22			
	Peptone, mg*			Peptone, mg*	
3.0	50	7	0.1	10	—
3.0	100	6	0.1	50	25
			0.1	100	21

* In addition to the 2.5 mg of pyridoxine in 25 cc of the original medium.

† Fungus growth reported on 50 ml basis.

TABLE IV.

Fungicidal Action of Clavacin and Actinomycin upon a Suspension of *C. ulmi* in Water.

Antibiotic substance			Viability		
Nature	Amt added, mg	Peptone, mg	1 day	2 days	No. of fungus cells in 1 ml 4 days incubation
0		0	+++	+++	2,800,000
Clavacin	1.5	0	++	0	0
,,	1.5	50	+++	+	30,000
,,	3.0	0	0	0	0
,,	3.0	50	++	Tr	16,000
,,	3.0	100	++	Tr	35,000
Actinomycin	0.1	0	+++	Tr	650,000
,,	0.1	50	+++	+++	4,850,000
,,	0	50	+++	+++	4,920,000

TABLE V.
Fungicidal Action of Clavacin and Actinomycin upon Growing Cultures of *C. ulmi* in Pyridoxine Media.*

Antibiotic substance			
Nature	Amt added,† mg	Additional pyridoxine, μ mg	*C. ulmi* cells in 1 ml
Actinomycin	0.1	0	2,570,000
”	0.1	5	7,750,000
Clavacin	1.5	0	1,000,000
”	1.5	2.5	1,200,000
”	1.5	5.0	1,500,000

* Materials added to 3-day-old cultures and plated after 2 days further incubation.

† To 25 ml of culture.

tone tends to overcome the activity of actinomycin to a greater extent than that of clavacin.

It is of interest to report here an unpublished observation,[‡] made during a study of the effect of vitamins upon the antibacterial activity of actinomycin, that vitamin C was the only one that exerted a partial inhibiting effect upon this substance.

Summary. A study has been made of the fungistatic and fungicidal action of various antibiotic substances upon *Ceratostomella ulmi,* the causative agent of the Dutch elm disease. Actinomycin and clavacin had a strong effect upon this fungus, penicillin and streptothricin had no effect at all, and other substances fell between.

The antifungal activities of clavacin and especially of actinomycin could be partly overcome by certain nutrients in the medium, especially peptone. Whether this is due to the pyridoxine content, to some other neutralizing effect or to a nutrient effect of the peptone is still to be determined.

[‡] This observation was made by Dr. H. Boyd Woodruff working in our laboratory.

14311

Protein Fractionation and Separation in the Large Tiselius Cell.*

C. L. San Clemente. (Introduced by E. E. Ecker.)

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The Tiselius electrophoresis apparatus introduced to America about 1937 is now commonly found in the leading laboratories throughout the country. The War has focused much effort upon protein solutions, especially serum and plasma, and as a result the Tiselius apparatus found not only immediate but intense application.

In order to separate the fractions obtained by electrophoresis, Tiselius[1,2] has used 2 small so-called separation cells. A compensator mechanism made it possible to move mechanically the total protein solution until a desired fraction was concentrated within the upper or lower channel. One or both of the middle sections were slid out of alignment. The supernatant buffer was removed, and finally the fractions in the various compartments were obtained individually by means of an extended steel needle attached to a syringe. Longsworth[3] recently has improved the "convection-proof" pipet technic of Tiselius[4] by lowering a fine glass capillary into the cell and very carefully withdrawing a desired fraction with a fixed syringe-compensator device. The removal of any fraction could be accurately controlled by direct Schlieren observation.

The method of Tiselius is satisfactory but causes undue dilution of the desired component, while the Longsworth technic requires additional apparatus and extreme

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* Aided by a grant from the Commonwealth Fund.

[1] Tiselius, A., *Trans. Faraday Soc.,* 1937, **33**, 524.

[2] Tiselius, A., *Biochem. J.,* 1937, **31**, 1464.

[3] Longsworth, L. G., *Chem. Rev.,* 1942, **30**, 323.

[4] Tiselius, A., *Kolloid-Z.,* 1938, **85**, 129.

care, if diffusion is to be avoided.

This note describes a method to separate fractions in the single, large middle section of the Tiselius cell without extra equipment nor danger of diffusion. For example, fresh serum was diluted 1:2.5 with buffer and dialyzed one day in 2 liters of the same buffer. Electrophoresis was carried out in the usual way until the main components were separated almost completely from each other over two-thirds of the length of the channel. The middle section was moved to one side. The glass plug on the side arm of the "open" electrode vessel was removed, and all the buffer above the cell and from part of the main vessel was siphoned off. The middle section was slid back in place again; no movement of solution occurred because of the "closed" electrode vessel. The 3-way stopcock on the side arm of the "closed" electrode vessel was turned to permit continuity between the vessel and compensator syringe. The compensator motor was started and the topmost component was pushed slowly into the empty side arm of the "open" electrode vessel. The progress of this movement was followed easily by direct observation on the ground glass plate. When this first component was just completely above the glass flanges of the cell, the motor was stopped and the middle section was moved out of alignment. A long needle on a syringe was used to remove the freed component. After rinsing the side arm, the middle section was realigned, and each of the components was separated in the same manner.

The advantages of the above technic include: (1) simplicity and rapidity; (2) elimination of special extra equipment; (3) avoidance of dilution beyond that which has occurred from the migration of the respective component; (4) minimizes the danger of diffusion between components. It is true that a small amount of overlapping occurs between fractions but this factor may be eliminated by combining the respective components from several runs of whole material, followed by electrophoresis in the same manner of each of the components separately. The small amount of faster and slower moving material traveling with the main component can now be "cut" off without much difficulty.

14312

Influence of Incubation at 37° C on Stability of Thiamin and Riboflavin in Cow's Milk.*

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Our recent finding[1] that animal tissues destroy appreciable amounts of thiamin and riboflavin during incubation at 37°C for 24 to 48 hours suggested a study of the stability of these vitamins in cow's milk under similar treatment. Pasteurized cow's milk, obtained from the University Creamery, was used throughout this investigation. Similar results, however, were obtained with raw untreated milk.

The experiments were carried out with 20 cc samples of milk, which contained 4.95 to 7.7 μg thiamin and 29 to 32 μg riboflavin. In the first experiment, 4 20 cc samples of cow's milk, to which have been added 0 μg, 10 μg, 100 μg and 1,000 μg of thiamin and equal doses of riboflavin, were incubated at 37°C for 48 hours. It will be noted from Table I that there was considerable destruction of thiamin in cow's milk during this

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* Research paper No. 776, Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

[1] Sure, B., and Ford, Z. W., *J. Nutr.*, 1943, in press.

TABLE I.
Influence of Incubation at 37°C for 48 Hours on the Stability of Thiamin and Riboflavin in 20 cc Samples of Cow's Milk.

Thiamin added, μg	Thiamin incubated, μg	Thiamin recovered, μg	% thiamin destroyed	Riboflavin added, μg	Riboflavin incubated, μg	Riboflavin recovered, μg	% riboflavin destroyed
—	7.7	2.69	65.0	—	31.67	30.85	2.6
10	17.7	7.20	59.3	10	41.67	40.83	2.0
100	117.7	82.0	30.5	100	131.67	125.25	4.9
1,000	1,017.7	690.0	32.2	1,000	1,031.67	1,032.50	0.0

TABLE II.
Influence of Previous Heat Treatment Followed by Autoclaving for 20 Minutes at 15 lbs. Pressure on Stability of Thiamin and Riboflavin to Incubation at 37° C for 22 Hours. Samples 20 cc of Milk.

Treatment	Thiamin incubated, μg	Thiamin recovered, μg	% thiamin destroyed	Riboflavin incubated, μg	Riboflavin recovered, μg	% riboflavin destroyed
Incubated	4.95	2.70	45.5	27.50	27.50	0.0
Heated to boiling and then incubated and autoclaved		2.67	46.0		27.50	0.0
Boiled 10 min. and then incubated and autoclaved		2.62	47.0		27.50	0.0
Boiled 20 min. and then incubated and autoclaved		1.23	75.7		25.00	9.1

period of incubation but that only negligible amounts of riboflavin were destroyed. These results suggested the presence in cow's milk of a factor or factors that destroyed thiamin during incubation at body temperature for a period of 2 days.

In order to determine the heat stability of such factor or factors, another series of experiments were carried out, in which the milk was brought to a boil, boiled for 10 minutes, and for 20 minutes before proceeding with analyses.

Since incubation curdled the milk, it was necessary to extract each sample and incubate with takadiastase before completing the thiamin determinations. In the first experiment (Table I) 75 cc of N/10 HCl were added to each sample, autoclaved for 20 minutes at 15 lbs pressure, incubated for 2 hours and analyzed according to the procedure of Hennessy and Cerecedo,[2] using the Merck and Co. modifications.[3] In the second experiment (Table II) the milk samples were given the same treatment but, in addition, were subjected to boiling temperature for various periods previous to autoclaving and incubation. From these results it is evident that incubation of cow's milk, containing about 5 μg thiamin and 28 μg riboflavin, at 37°C for 22 hours results in approximately 46% destruction of thiamin but that riboflavin is 100% stable under such treatment; also boiling for 10 minutes produced no change. Boiling for 20 minutes, however, produced an additional 30% destruction. From these results it would appear that the factor or factors in cow's milk responsible for thiamin destruction at 37°C is heat stable, and, therefore, probably non-enzymic in nature. On the other hand, the increased destruction of thiamin following 20 minutes of boiling is due to instability of this vitamin to heat treatment at 100°C. The latter procedure also produced small destruction of riboflavin.

Allowing 20 cc cow's milk, containing 6 μg thiamin, to stand at room temperature of 22°C for one week, produced only 6% destruction of thiamin. Allowing duplicate samples of milk to stand in a room at a tem-

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[2] Hennessy, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 1939, **61**, 179.

[3] Merck and Company, Inc., *Determination of Thiamin Hydrochloride by the Thiochrome Method, with the Adaptation of the Hennessy and Cerecedo Procedure*, revised June 6, 1941.

TABLE III.
Influence of Ash of Milk and pH on Stability of Thiamin to Incubation at 37°C for 22 Hours.

Thiamin, µg	Treatment	Thiamin recovered, µg	% thiamin destroyed
6	In 20 cc water, pH 5	5.73	4.5
6	In 20 cc water + ash of 20 cc cow's milk	4.93	17.83
6	In 20 cc water, pH 6	5.60	6.67
6	In 20 cc water, pH 6, + ash of 20 cc cow's milk	4.87	18.83
6	In 20 cc water, pH 7	5.33	11.17
6	In 20 cc water, pH 7, + ash of 20 cc cow's milk	4.67	22.17

perature of 31°C resulted in 16% destruction. Since incubation at 37°C for 22 hours produced 46% destruction, the critical temperature of thiamin destruction in cow's milk seems to be between 31 and 37°C.

The next point of interest that seemed worthy of investigation was to determine what contribution, if any, the minerals of milk made towards the thiamin destruction at body temperature. The results of such trials are presented in Table III. It will be noted there is only a slight destruction of thiamin in aqueous solution which increases with the increase of the pH from 5 to 7. However, the ash of milk *per se* produced a destruction of 11 to 12% thiamin, regardless of the pH. In other words, the minerals of milk are responsible for about 25% of the total thiamin destruction; therefore, the character of the factor or factors which are contributory to 75% of the destruction must be organic in character and of a nature that resist heat treatment at 100°C for 10 minutes.

Summary. Incubating cow's milk for 22 hours at 37°C produces 46% destruction of thiamin but riboflavin is entirely stable under such treatment. The critical temperature for thiamin destruction appears to be between 31 and 37°C. The factors in cow's milk responsible for thiamin destruction are stable to 100°C for 10 minutes. The minerals of cow's milk are contributory to 25% of the thiamin destroyed.

14313

Effect of Certain Organic Compounds on Germicidal Efficiency of Mercuric Chloride.

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In practice disinfectants are commonly used in the presence of organic matter. The value of the results obtained by any laboratory procedure may justly be questioned if the comparisons are made in the absence of organic matter. Despite the significance of this fact, which was realized and emphasized by Chick and Martin,[1] most tests for rating germicides are still performed in the absence of organic compounds.

It is well known that the type of organic matter employed may make a big difference in the final results obtained. Some compounds are without effect whereas others influence markedly the efficiency of germicides. Unless a standard organic solution or compound is incorporated in the procedure employed for the evaluation of germicides the final results will give misleading comparisons.

The present investigation was undertaken to determine which groups of organic compounds are effective in reducing the germicidal efficiency of mercuric chloride. Definite amounts of gelatin, peptone, a number of amino acids, and several carbohydrates were employed. The concentration of mercuric chloride required to kill *Staphylococcus aureus* in the presence of definite amounts of these organic compounds was determined and the results compared with a control series in which no organic matter was employed.

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[1] Chick, H., and Martin, C. J., *J. Hyg.*, 1908, **8**, 654.

Method. To each of a series of tubes, containing 2.5 cc of organic matter adjusted to pH 6.6, was added 0.5 cc of a 24-hour broth culture of *Staphylococcus aureus.* The tubes were brought to 37°C in a water bath.

A series of aqueous dilutions of mercuric chloride were prepared and brought to 37°C. Two cc of each dilution of the germicide were pipetted into the tubes containing the mixture of bacteria and organic matter, bringing the final volume to 5 cc. The contents of the tubes were mixed thoroughly.

TABLE I.
Effect of Carbohydrates on the Germicidal Efficiency of Mercuric Chloride Against *Staphylococcus aureus.*

Dilution of $HgCl_2$	Control	Glucose	Maltose	Sucrose
1:12,500	—	—	—	—
1:25,000	+	+	+	+
1:37,500	+	+	+	+
1:50,000	+	+	+	+

— = no growth; + = growth after 96 hours at 37°C.

After 10 minutes the tubes were removed from the water bath and a 4 mm loopful transferred from each tube to broth. To eliminate a bacteriostatic effect, subcultures were made to 100-cc amounts of broth contained in flasks. The killing dilution was determined by the absence of growth in the subcultures after an incubation period of 96 hours at 37°C.

Controls were run for each series, using 2.5 cc of distilled water in place of the organic matter.

Effect of Carbohydrates. Glucose, maltose, and sucrose were selected as being representative of the carbohydrates. Each sugar was dissolved in distilled water to give a concentration of 2%. The solutions were sterilized by passage through Chamberland filters.

The results are given in Table I. The figures show that the 3 carbohydrates tested had no effect on the destruction of *Staphylococcus aureus* by mercuric chloride. The highest dilution of germicide showing no growth of the organism was 1:12,500 regardless of whether the sugars were present or absent.

Effect of Amino Acids. Glycine, cystein, aspartic acid, glutamic acid, arginine, and lysine were dissolved in distilled water to produce solutions of M/15 concentrations, then sterilized. With the exception of cystein, the acids were sterilized in the autoclave at 15 lb pressure for 30 minutes. Since cystein in the presence of free oxygen, traces of iron, and heat is rapidly converted into insoluble cystine, it was sterilized by passage through a Chamberland filter and used immediately.

The results are reported in Table II. The amino acids produced considerable inactivation of the mercuric chloride. With the exception of cystein, the acids showed the same degree of inactivation of the germicide. In the presence of the amino acids, a dilution of 1:2500 of mercuric chloride was required to kill the test organism; in the absence of the acids a dilution of 1:12,500 produced the same result. Cystein was an exception in that it showed considerably more inactivation than the other amino acids. In its presence mercuric chloride failed to kill the test organism in a dilution of 1:250, the highest concentration used.

It is generally stated that neutral salts of the heavy metals react with amino groups to form double salts of definite composition. If this is true the dibasic acids (arginine, lysine) should produce twice as much inactivation of mercuric chloride as the monobasic acids. Such was not the case, however, as all of the acids except cystein produced equal inactivation of the germicide when employed in equimolar concentrations. No explanation is advanced for this result.

On the other hand cystein contains a sulfhydryl (—SH) group that is also capable of reacting with mercury.[2] This leads to a further inactivation of mercury with the result that more mercuric chloride is required to produce the same toxic effect on the bacteria.

Effect of Aminoids, Peptone, and Gelatin. One per cent solutions were prepared in

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[2] Fildes, Paul, *Brit. J. Exp. Path.*, 1940, **21**, 67.

TABLE II.

Effect of Amino Acids on the Germicidal Efficiency of Mercuric Chloride Against *Staphylococcus aureus.*

Dilution of $HgCl_2$	Control	Glycine	Aspartic acid	Glutamic acid	Arginine	Lysine	Cystein
1:250	—	—	—	—	—	—	+
1:500	—	—	—	—	—	—	+
1:1000	—	—	—	—	—	—	+
1:2500	—	—	—	—	—	—	+
1:5000	—	+	+	+	+	+	+
1:7500	—	+	+	+	+	+	+
1:12,500	—	+	+	+	+	+	+
1:25,000	+	+	+	+	+	+	+
1:37,500	+	+	+	+	+	+	+

— = no growth; + = growth after 96 hours at 37°C.

distilled water, the solutions adjusted to pH 6.6, and sterilized in the autoclave. The results are recorded in Table III. Aminoids caused more inactivation than peptone and the latter more than gelatin. In view of the fact that Aminoids contains more highly hydrolyzed protein than peptone and, therefore, possesses more reactive groups per unit weight, the results are according to expectations.

TABLE III.

Effect of Aminoids, Peptone, and Gelatin on the Germicidal Efficiency of Mercuric Chloride Against *Staphylococcus aureus.*

Dilution of $HgCl_2$	Control	Aminoids	Peptone	Gelatin
1:2500	—	—	—	—
1:5000	—	+	—	—
1:7500	—	+	+	—
1:12,500	—	+	+	+
1:25,000	+	+	+	+

— = no growth; + = growth after 96 hours at 37°C.

Summary. The carbohydrates glucose, maltose, and sucrose produced no effect on the germicidal efficiency of mercuric chloride. The concentration required to kill *Staphylococcus aureus* was the same whether the sugars were present or absent.

The amino acids glycine, aspartic acid, glutamic acid, arginine, lysine, and cystein produced considerable inactivation of mercuric chloride. With the exception of cystein the acids produced the same degree of inactivation when employed in equimolar concentrations. Cystein produced more inactivation than the other acids because of the presence of a sulfhydryl group which is also capable of reacting with mercury.

Gelatin, Aminoids, and peptone, also produced considerable inactivation of mercuric chloride. The degree of inactivation was found to parallel the number of free amino groups present. The results show that the inactivation of mercuric chloride was dependent upon the presence of proteins, protein split products, and amino acids. Carbohydrates were without effect.

14314

The Rectal Administration of Dicumarol.*

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It has been demonstrated that Dicumarol, 3,3′-methylenebis (4-hydroxycoumarin), synthesized by Link and his associates,[1] can be successfully administered to human beings orally, and intravenously as the disodium salt in an alkaline solution of pH 10 or slightly higher. Upon administration of the drug in proper dosage—5 mg/kg—by either route there is, in 80% or more of individuals, a significant prolongation of the prothrombin time following a latent period of 24 hours or more. Both methods, however, have their limitations: it is often undesirable to apply oral therapy, as after certain operations, and the intravenous solution is not very stable. Hence the rectal administration of the drug would be advantageous if good absorption and a satisfactory decrease in the prothrombin time and coagulability of the blood could be achieved by this method.

The feasibility of the rectal administration of Dicumarol was first tested in a 46-year-old male suffering from thrombosis of the right postero-inferior cerebellar artery and resultant inability to swallow. In this patient the effect was very satisfactory: the rectal administration of 3 mg/kg (258 mg) in a water suspension increased the prothrombin time within 2 days from a pre-administration level of 15.5 seconds to 25.5 seconds. A second dose of the same size 4 days later maintained the elevated prothrombin time for 6 days more. This was fully as satisfactory an effect as would be expected from a similar dose given orally.

Later 2 doses of the drug, incorporated in cocoa butter suppositories, 5.0 mg/kg, were administered, 5 days apart, to an 86-kg man of 65 afflicted with posterior inferior cerebellar artery thrombosis. In this patient the prothrombin time was increased from a control level of 11 seconds to a maximum of 42 seconds the second day after the second dose. The coagulation time was increased from 8 minutes to a maximum of 19 minutes on the seventh day after the second dose.

These two cases demonstrate that Dicumarol may be effective when administered rectally in aqueous suspension or in a suppository. However, in only 2 of 34 other patients with a variety of diseases to whom the drug was administered rectally in doses of 5, 7.5, and 10 mg/kg in aqueous suspension or suppositories (11 of the 34 received the largest dose) was there a significant decrease in the coagulability of the blood, and prothrombin.

Discussion. It is of interest that Dicumarol can, on occasion, be absorbed when administered rectally and produce effects similar to those resulting from oral or intravenous exhibition of the drug. When it is effective, the latent period is no longer than when the Dicumarol is given orally.

Conclusion. The administration of Dicumarol rectally in aqueous suspension or in cocoa butter suppositories is only occasionally effective; hence its routine employment cannot be relied upon.

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* This study was aided in part by a grant from the Wisconsin Alumni Research Foundation.

[1] Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 513.

14315

Lipotropic Action of Inositol.

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It has been shown by Gavin and McHenry[1] that the fatty livers which result from the administration of biotin to rats on a high carbohydrate-low protein diet are very resistant to the lipotropic action of choline but respond readily when inositol is given in addition. A recent publication by McHenry and his associates[2] shows that inositol is apparently inactive against the fatty livers which develop when thiamine is the only member of the B-vitamins fed, however, when riboflavin and pyridoxin, or these plus additional B vitamins are given in addition to thiamine, inositol is definitely lipotropic. Engel[3] also has shown that inositol exerts a lipotropic effect in the absence of biotin in the food. The results obtained in the present investigation are in good agreement with those of the above authors.

In order to control scattering of food, especially during the deficiency period, the food was made up as a concentrated aqueous solution containing insoluble material in suspension. This diet had the following composition:

Casein, vitamin free, Labco brand	120	g
Sucrose	1200	”
Salt mixture (Wesson)[4]	36	”
Cellu flour B*	12	”
Percomorph oil, 50%	0.5	cc
Water, distilled	960	”
Vitamin B-complex as given in text		

Experimental. White rats weighing 70-90 g were put in individual cages and fed daily 10-15 cc of the above diet, supplemented with 3 mg of thiamine chloride per 1200 g of sucrose. A 15 cc Folin and Wu pipet with a broken tip was used for measuring it. The amount given was so regulated that it was completely, or almost completely, consumed each day. After the animals were on this deficient diet for 3 weeks they were put on

TABLE I. Neutral Fat and Cholesterol Content of Liver, Body, and Skin.

Exp. No.	1			2		3		4		5		6			7		
Thiamine chloride	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Riboflavin		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyridoxin chloride		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ca pantothenate		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Niacin				+	+					+	+	+	+	+	+	+	+
Choline chloride, mg*												1		1	5		5
Inositol mg*			10		20		20		10		20		10	10		5	5
No. of rats	2	4	4	4	4	4	5	5	5	5	5	3	3	4	4	2	4
Daily avg gain wt, g	0.6	1.6	1.9	1.4	1.8	1.8	1.9	2.3	2.5	0.6	0.6	1.9	1.6	2.4	1.8	1.9	2.1
Avg liver wt, g	4.75	5.25	5.18	6.76	5.66	5.32	5.26	5.40	5.20	5.50	5.00	6.05	5.10	4.73	4.98	5.88	4.62
Liver neutral fat†	9.2	9.8	7.1	18.7	9.5	11.1	3.5	7.7	7.1	10.4	6.4	20.9	11.0	5.8	9.9	16.2	4.1
Liver cholesterol	.45	.56	.45	.73	.45	.54	.33	.74	.48	.61	.54	.82	.73	.48	.55	.55	.56
Body neutral fat†	5.5	7.7	8.3	6.2	8.5	6.6	6.7	9.8	9.8	6.0	6.3	8.8	8.7	9.4	8.5	8.9	9.9
Body cholesterol†	.18	.16	.19	.15	.19	.18	.18	.22	.22	.19	.21	.17	.17	.16	.18	.17	.18
Skin neutral fat†						14.8	15.2	21.3	21.3	13.2	14.7	17.0	15.6	20.4	17.2	17.0	21.3
Skin cholesterol†						.21	.22	.18	.19	.27	.31	.17	.16	.14	.19	.18	.20

* Mg per 10 g of sucrose in diet. † % wet weight. All animals in experiments 3, 4 and 5 received 0.5 γ of biotin per 10 g of sucrose in the diet during the supplemental feeding periods.

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[1] Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, 1941, **139**, 485.

[2] Gavin, G., Patterson, J. M., and McHenry, E. W., *J. Biol. Chem.*, 1943, **148**, 275.

[3] Engel, R. W., *J. Nutrition*, 1942, **24**, 175.

[4] Wesson, L. G., *Science*, 1932, **75**, 339.

* Obtained from Chicago Dietetic Supply House, Chicago.

additional supplements for a period of 9-11 days, following which the animals were sacrificed for lipid analyses. These supplements were always given at the following levels, riboflavin 9 mg, pyridoxine hydrochloride 6 mg, calcium pantothenate 21 mg, niacin 21 mg, and biotin 60 γ per 1200 g of sucrose. Choline chloride and inositol were administered as recorded in the accompanying table.

The animals were rapidly exsanguinated by decapitation at the end of the supplemental feeding period. Their livers were removed, weighed, and after grinding in a mortar, analyzed individually for neutral fat and cholesterol. The intestinal tract was removed and the animal skinned, after which the neutral fat and cholesterol content of both the bodies and skins from each group was collectively determined. The analytical procedures have been described previously.[5,6]

From the experimental results, which are recorded in Table I, it will be seen that the lipotropic action of inositol in the absence of biotin from the diet is confirmed. The effect, however, is much less than one would expect from choline under the same conditions. The effect of choline plus inositol is greater than of either alone but the data are not sufficient to show whether they act synergistically or not. The effect on liver cholesterol is, on the whole, less than on the neutral fat. The small amount of biotin used in experiments 3, 4 and 5 did not apparently influence the lipotropic action of the inositol, neither did it increase the lipid content of the livers in the absence of inositol. These results are contrary to those obtained by McHenry and his associates[1] but it is possible that if we had used larger amounts of biotin, choline-resistant inositol-sensitive fatty livers might have been obtained.

The neutral fat content of the body and skin of those animals receiving both choline and inositol is greater than that of the corresponding animals receiving these individually. Whether this is due to an increased food intake, as suggested by the greater rate of growth of these animals, or to a stimulating effect on fat synthesis, cannot be stated at this time as food intake was not sufficiently accurately controlled to justify definite conclusions.

Summary. The lipotropic action of inositol in the presence of other members of the vitamin B-complex has been confirmed. The effect of inositol plus choline is considerably greater than that of either alone.

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[5] Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, 1940, **25**, 1157.

[6] Forbes, J. C., *J. Nutrition*, 1941, **22**, 359.

14316 P

Preparation of the Pituitary Corticotrophic Hormone.*

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The procedure used for the preparation of the corticotrophic hormone from whole and from dissected calf anterior pituitary tissue was based on methods recommended by Lyons, by Li, Simpson and Evans, and by Sayers, White and Long.[1-3] It has been our experience that most of the posterior lobe potency present in the starting material remains with the fraction showing corticotrophic activity. Attempts made to separate these factors by their solubilities at various pH and salt concentrations were without success. The same held true in utilizing dialysis

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* Aided by a grant from the Dazian Foundation.

[1] Lyons, W. R., Proc. Soc. Exp. Biol. and Med., 1937, **35**, 645.

[2] Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, 1942, **96**, 450.

[3] Sayers, G., White, A., and Long, C. N. H., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 199.

TABLE I.
The Assay Results of the Pituitary Corticotrophic Preparations.

Preparation	NH_4OH temp.-time, hr	Amt given daily, mg	Wt of single adrenals; in brackets—left, control adrenal, mg	Oxytocin μ/mg	Anti-diuretic μ/mg
Step 5—ant. pit.	—	0.1	7 (5); 7 (4); 6 (5); 5 (4)	.04-.07	.05-.09
		0.2	9 (6); 8 (5); 9 (5); 7 (5)		
		0.25	15 (7); 13 (7); 12 (6); 16 (7); 11 (7); 10 (7)		
		0.5	9 (6); 15 (7); 13 (6); 16 (5); 11(6); 18 (5)		
Step 5—whole pit.	—			3.8-4.0	3.3-3.9
Step 7—ant. pit.	37°, 4	0.25	6 (4); 7 (3)	.02-.03	.02-.04
		0.5	18 (9); 7 (3); 10 (4); 11 (7); 16 (8); 12 (7)		
		1.0	19 (5); 16 (6); 12 (5); 13 (6); 12(7); 21 (6)		
Step 7—ant. pit.	37°, 10	0.25	9 (5); 11(7)	.002-.003	.002-.003
		0.5	12 (8); 15 (7); 11 (6); 8 (5); 9 (6)		
		1.0	15 (7); 18 (6); 16 (6); 11 (5); 12 (7)		
		2.0	16 (5); 19 (6); 21 (8); 14 (6)		
Step 7—whole pit.	37°, 10	0.25	7 (6); 8 (5); 7 (3); 9 (6)	.12-.15	.12-.16
		0.5	10 (6); 14 (5); 9 (6); 8 (5); 10 (4)		
		1.0	16 (4); 17 (7); 12 (7); 15 (5); 14 (6)		
Step 7—ant. pit.	25°, 6	0.5	12 (7); 10 (6); 9 (5); 9 (6); 12 (5)	.02-.04	.03-.04
		1.0	16 (7); 18 (6); 12 (5); 16 (5); 16 (4)		
Step 7—ant. pit.	25°, 14	0.25	8 (6); 12 (7); 6 (5); 8 (5)	.003-.005	.004-.006
		0.50	10 (6); 14 (8); 9 (5); 16 (6); 10 (4)		
		1.0	12 (5); 17 (7); 17 (4); 13 (5); 12 (6); 18 (5)		
Step 7—whole pit.	25°, 14	0.5	12 (6); 18 (5); 11 (6); 16 (4); 14 (8)	.18-.19	.17-.19
		1.0	18 (6); 21 (4); 23 (8); 16 (8); 13 (4)		

with membranes of various porosities. This led us to attempt inactivation of the posterior lobe hormones with alkali.[4] Treatment with NaOH under varying conditions of concentration and time, when successful in removing posterior lobe activities, almost invariably resulted in appreciable losses of corticotrophic potency. The same held true for NH_4OH treatment at higher temperatures. NH_4OH treatment at 37° and at 25°C resulted in the inactivation of most of the posterior lobe hormones without any appreciable loss of corticotrophic activity (Table I).

The method utilized for the preparation of the corticotrophic hormone containing only traces of posterior lobe contamination was as follows: (1) An 80% acidified acetone extraction was made from thoroughly minced tissue. Two such extractions, 6 hours each, were sufficient to remove all the active material; the residues being subjected to pressures in each instance. This was carried out at room temperatures, all subsequent procedures were done at 0° to 3°C. (2) The active material was precipitated from the combined filtrate by raising the acetone concentration to 90%. This was thoroughly washed with cold acetone and dried. (3) The powder, containing the corticotrophic and prolactin hormones, was extracted repeatedly with 0.1 M Na_2HPO_4. This was brought to half saturation with $(NH_4)_2SO_4$, the precipitate formed taken up in water and dialyzed. (4) The dialyzed solution (made to a concentration of 0.5%) was adjusted to pH 3 and NaCl added to 0.36 M. The precipitate formed, containing the prolactin activity, can be further purified. The filtrate was brought to half saturation with $(NH_4)_2SO_4$, filtered, taken up in water and dialyzed. (5) The pH was adjusted successively to 8.0, 6.6, and 5.4 and the precipitates formed discarded. (6) One-half volume of concentrated NH_4OH was added and this allowed to stand at 37° or 25°C for varying lengths of time (Table I). Then acetone was added to a concentration of 90%, the precipitate formed taken up in water and dialyzed. (7) Step 5 was repeated, the final solution dialyzed until free of salt and lyophilyzed. The yield of material has varied between 0.6 to 1.2 g per kg (with whole glands the yield was 0.6 to 0.88 g, with dissected anterior lobes, 0.76 to 1.2 g).

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[4] Collip, J. B., *Edinburgh Med. J.*, 1938, **45**, 782.

When assayed for growth, prolactin, thyreotrophic and gonadotrophic activities this material showed negative results with the amounts used. The assays for corticotrophic activity were done on young rats weighing 100 to 120 g, 14 to 18 days after hypophysectomy. The left adrenal was removed and weighed prior to injection. Daily injections were given for 9 days and the right adrenal weighed on the 10th day. The daily amounts of the final material necessary to restore the right adrenal size to normal ranged between 0.25 and 0.5 mg (Table I). This is of the same order of potency as that reported by Li *et al.* and Sayers *et al.* The preparations made from whole glands showed a similar corticotrophic potency, but still retained appreciable posterior lobe contamination (more than 0.1 μ per mg).

14317 P

Congenital Malformations Induced in Rats by Maternal Nutritional Deficiency. V. Effects of a Purified Diet Lacking Riboflavin.

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The appearance of congenital malformations in the offspring of female rats fed a deficient diet (Diet I) has been described.[1,2] Diet I had the following percentage composition: Yellow corn meal 76, wheat gluten 20, calcium carbonate C.P. 3, and sodium chloride C.P. 1. Sixty International Units of vitamin D as viosterol were administered to each female by pipette every tenth day. The malformations could be prevented in the offspring when the maternal diet was supplemented by 2% dried pig liver.[3] It was concluded that a substance deficient in Diet I and present in large amounts in liver was responsible for the presence or absence of the congenital defects.

Several investigators have reported satisfactory growth and reproduction of rats on highly purified diets in which synthetic factors of the vitamin B-complex (thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and choline) were employed.[4-7]

We have studied the reproduction of female rats fed a similar purified diet which, however, did not contain riboflavin.

Twenty-one female rats of the Sprague-

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[1] Warkany, J., and Nelson, R. C., *Science*, 1940, **92**, 383.

[2] Warkany, J., and Nelson, R. C., *Anat. Rec.*, 1941, **79**, 83.

[3] Warkany, J., and Nelson, R. C., *J. Nutrition*, 1942, **23**, 321.

[4] Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 625.

[5] Unna, K., *Am. J. Med. Sci.*, 1940, **200**, 848.

[6] Unna, K., Richards, G. V., and Sampson, W. L., *J. Nutrition*, 1941, **22**, 553.

[7] Henderson, L. M., McIntire, J. M., Waisman, H. A., and Elvehjem, C. A., *J. Nutrition*, 1942, **23**, 47.

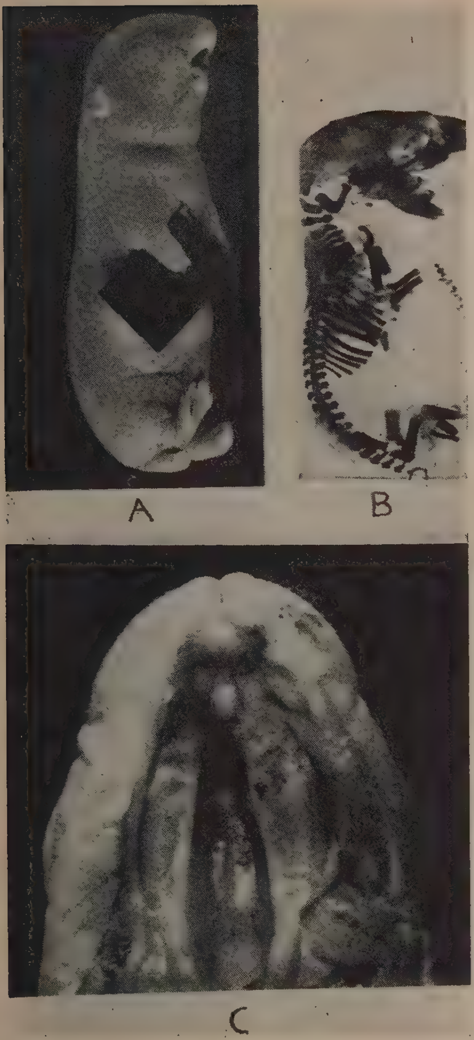

Fig. 1 A.
Abnormal newborn rat showing short mandible, protruding tongue, short extremities, and syndactylism of the hand.

1 B.
Cleared specimen of an abnormal newborn rat showing short mandible and short tibia, and fusion of ribs.

1 C.
Cleft palate of an abnormal newborn rat.

Dawley strain, reared on an adequate diet and weighing from 170-240 g were put, at the age of 3 months, on a purified diet of the following percentage composition: Sucrose 68, Vitamin-test Casein 18, Vegetable Oil 10,[*] Salt Mixture 4.[†] One hundred grams of this diet were supplemented by 0.8 mg thiamine hydrochloride, 0.8 mg pyridoxine hydrochloride, 1 mg calcium pantothenate, 10 mg nicotinamide (Merck), and 100 mg choline chloride (SMACO). Every tenth day 1-2 drops of a 1:4 dilution with olive oil of "Drisdol with vitamin A in oil" (Winthrop) were administered by pipette.

During the first 2 weeks the rats maintained their weight on this diet or gained from 4 to 15 g. They also showed regular estrous cycles for several weeks. They were mated to normal males of the same strain which had been fed an adequate stock diet. All but 2 of the females became pregnant within 3 weeks. Of these 19 pregnancies 11 ended in resorption while 8 females delivered their litters at term. The pregnancies caused such great weight losses in 11 animals that they were considered unfit for further breeding. Of the 8 rats that remained in the experiment 4 did not show estrous cycles within 8 weeks after the first pregnancy. Four rats underwent a second pregnancy. One of these resorbed, while 3 delivered their litters at term.

A total of 74 young was obtained from these female rats fed the purified diet lacking riboflavin. Sixty-four of these young were anatomically normal on external inspection and after clearing. Ten of the newborn rats showed congenital malformations of the "pattern of Diet I"[8] (Fig. 1A and B). Three of the abnormal young were the result of a first pregnancy and 7 the result of 2 second pregnancies. The malformations consisted in shortness of the mandible, tibia, fibula, radius and ulna, fusion of ribs and sternal centers of ossification, syndactylism, brachydactylism and cleft palate (Fig. 1C). The frequency of the osseous defects in the 10 abnormal specimens is indicated in the following tabulation:

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[*] The mixture of sucrose, casein, and vegetable oil was furnished by S. M. A. Corporation.

[†] Salt Mixture No. 351 as recommended by Hubbel, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, **14**, 273.

[8] Warkany, J., Nelson, R. C., and Schraffenberger, E., *Am. J. Dis. Child.*, 1943, **65**, 882.

Tibia	7	Hand	6
Mandible	8	Sternum	2
Ribs	6	Ulna	4
Fibula	5	Hindfoot	4
Radius	5	Cleft palate	2

One of the abnormal young showed in addition to the abnormalities of the "pattern of Diet I" a defect of the spine.

Summary. Congenital malformations were observed in the offspring of female rats fed a purified diet lacking riboflavin. The malformations were anatomically identical with those described previously as the "pattern of Diet I."

14318

Cephalin, Protamine and the Antithromboplastic Activity of Normal and Hemophilic Plasmas.

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Normal human plasma, collected and separated with especial precautions, has the property of reducing the clot accelerating activity of homologous brain tissue extracts. The clot decelerating activity of hemophilic plasma is several times greater than normal.[1] The thromboplastin of aqueous brain-tissue extracts, on which this activity of the plasma has been tested, is a lipoprotein; its clot accelerating properties reside chiefly in the lipid moiety, the active constituent of which is a cephalin.[2] From the evidence here presented it appears that cephalin is the constituent of the lipoprotein vulnerable to the antithromboplastic action of the plasma. When the lipoprotein is stripped of most of its lipid content by extraction with fat solvents, it loses most of its susceptibility to the antithromboplastin.

Methods for separating the plasma and testing antithromboplastin activity have been given in detail elsewhere.[1] Only clear, "platelet-free" plasmas may be used. The thromboplastic lipoprotein solution (Sol. A) was prepared by extracting 300 mg of acetone dried human brain powder with 5 cc of 0.85% NaCl.[3] Cephalin was obtained from acetone dried human brain by extraction at 5°C overnight, with ether of low peroxide content (1 g brain powder plus 20 cc ethyl ether). In view of the rapid loss of potency of cephalin solutions[4] the time of extraction was made short and only a small amount was prepared each time. The clear ether extract was rapidly concentrated *in vacuo* and poured into ice cold absolute ethanol. The white flaky precipitate was then washed with acetone, dried to a waxy consistency and suspended at once in 10 cc of saline. In a few instances the cephalin was purified further by repeating the passage of the lipid through absolute alcohol 2 or more times before suspending it in saline. A pale bluish grey suspension was obtained (Sol. B), 0.1 cc of which gave a clotting time between 60 and 80 seconds on 0.3 cc recalcified citrated normal human plasma. As is well known, the cephalin obtained by ether extraction of the thromboplastic lipoprotein does not have the potency of the latter (0.1 cc of a concentrated aqueous extract of brain tissue clots 0.3 cc of recalcified human plasma in 14-15 seconds). To the dried residue that remained after the twice-repeated ether extraction of 1 g of brain powder, 10 cc of 0.85% NaCl was added (Sol. C). All extracts were allowed to stand until the coarse particles had settled; the supernatant fluid was used for the

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[1] Tocantins, L. M., *Am. J. Physiol.*, 1943, **139**, 265.

[2] Howell, W. H., *Am. J. Physiol.*, 1912, **31**, 1.

[3] Quick, A. J., *Science*, 1940, **92**, 113.

[4] McLean, J., *Am. J. Physiol.*, 1917, **43**, 586.

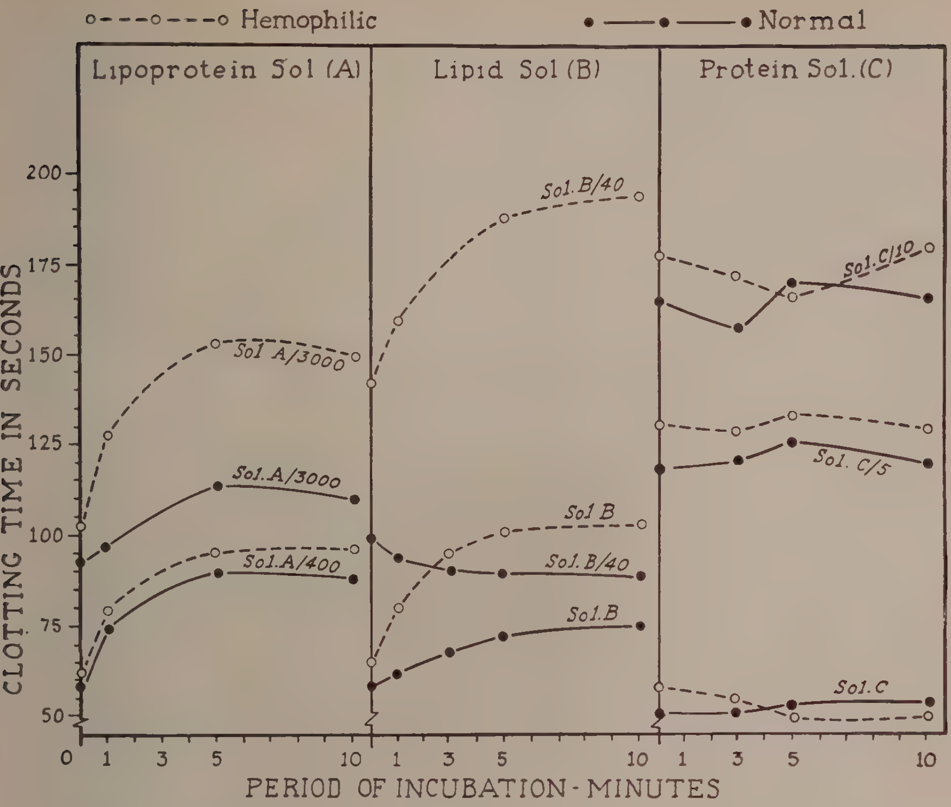

FIG. 1.

Effect of incubation of normal or hemophilic plasma with three types of clot accelerating solutions (Sols. A, B, or C). The numbers after each solution indicate its dilution.

0.3 cc plasma, 0.1 cc thrombopl. sol. (incubation), 0.1 cc $CaCl_2$. In the non-incubated mixtures, the calcium was placed in the tube first, the plasma and the thromboplastin solution immediately after. Mixing of the cephalin suspensions with the $CaCl_2$ before adding the plasma is to be avoided; flocculation of the cephalin that follows addition of the $CaCl_2$ interferes with the action of the lipid and so alters the structure of the clot as to make it difficult to determine the end point of coagulation.

tests, with appropriate dilution in order to obtain, on normal plasma, approximately similar times with the 3 clotting agents.

In Fig. 1 are compared the results of incubation of normal or hemophilic plasma with the thromboplastin lipoprotein or with either one of its 2 components, separately. A clear decelerating effect is observed with the thromboplastic lipoprotein or with the cephalin alone, but not with the protein alone. There is no further prolongation of the clotting time of the mixture even when the incubation is carried out for as long as 90 minutes. There is little difference between the effect of the protein alone on the 2 plasmas; this may be an indication that, since the thromboplastic protein does not seem to be vulnerable to the action of antithromboplastin, the latter is bypassed by the protein which acts directly on prothrombin. In this respect, the action of the thromboplastic protein resembles that of dilute Russel viper venom. This venom is likewise invulnerable to antithromboplastin[5] and acts equally well on normal and hemophilic blood.

It has been shown[1] that over-recalcifica-

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[5] Tocantins, L. M., *Fed. Proc.*, 1943, **2**, 48.

TABLE I.

The prothrombin time, plasma clotting time and anti-thromboplastic activity of plasmas (normal and hemophilic) to which protamine has been added. Non-incubated plasma mixtures handled as stated under Fig. 1. All plasmas tested within 10 minutes after adding the protamine to avoid the fibrinogenolysis[13] and rapid loss of antithromboplastic activity that follows standing at room temperature.

	Normal						Hemophilic					
	Control .85% NaCl	Mg of protamine per cc plasma					Control .85% NaCl	Mg of protamine per cc plasma				
		.025	.05	.1	.2	.4		.025	.05	.1	.2	.4
Prothrombin time (sec.)*	14	16	17	18	21	47	14	16	18	21	26	35
Plas. clot. time (min.)†	3.5	4.2	11	17	25	44	45	50	76	90	125	147
Plas. + thrombopl. + $CaCl_2$‡												
0′ incub. (sec.)	80	112	149	227	400	560	89	125	140	165	180	233
5′ incub. (sec.)	115	145	215	375	630	710	167	225	275	300	315	345
Plas. + cephalin + $CaCl_2$§												
0′ incub. (sec.)	67	82	112	158	184	297	146	290	400	425	525	650
5′ incub. (sec.)	89	106	136	172	228	430	233	325	447	520	568	720

* Quick[3] method.

† 0.3 cc plasma, 0.1 cc $CaCl_2$.

‡ 0.3 cc plasma, 0.1 cc dilute thromboplastin (incubation), 0.1 cc 0.074 M $CaCl_2$.

§ 0.3 cc plasma, 0.1 cc cephalin sol. (incubation), 0.1 cc 0.074 M $CaCl_2$.

tion, standing, heating (65°C for 5 min), dilution or previous incubation with a small amount of tissue extract reduce or eliminate the antithromboplastin activity of plasma. Similar results were obtained when cephalin instead of the thromboplastic lipoprotein was used. The clot decelerating effect of incubation of the plasma with the cephalin was irregular and often missing when heterologous cephalins (sheep, dog) were used on human plasma. This suggests that a certain degree of species specificity applies to the reaction between cephalin and antithromboplastin as it does between the latter and the thromboplastic lipoprotein.[1]

It appears, therefore, that the antithromboplastin activity of plasma is exerted on the cephalin moiety of the thromboplastic lipoprotein. Mills[6] and Eagle[7] have pointed out that hemophilic plasma is unusually resistant to activation by cephalin. A cephalin-like substance is responsible for the clot accelerating properties of platelets and platelet extracts.[8,9] These facts would seem to indicate that the excessive amount of antithromboplastin in hemophilic blood[1] is probably responsible for the resistance of this blood to activation by cephalin (or platelets) as well as for its delayed coagulability.

Chargaff[10] has demonstrated that certain basic proteins (protamines, histones) possess the property of forming true salts with cephalin (which is acidic). These lipoprotein complexes once formed are unusually resistant to disruption by various chemical means. The anticoagulating effect of protamine on the blood has been known for some time.[11] Part of this effect, at least, must be due to the direct neutralization of cephalin by the protamine which may therefore, as Chargaff[10] pointed out, be considered a true antithromboplastin. Plasma to which an appropriate amount of protamine is added should therefore behave towards thromboplastic tissue extract and cephalin essentially like hemophilic plasma. Addition of protamine (salmine sulfate) to normal plasma in the amounts designated in Table I does, indeed, lead to a prolongation of the plasma clotting time and the appearance of a degree of antithromboplastin activity closely resembling (in certain concentrations of the protamine) that observed in intact hemophilic plasma. Such protamine plasmas behave in other respects like plasmas with excessive amounts of antithromboplastin. Aging (24-48 hours at 5°C), dilution, over-recalcification, heating to 65°C (5′) or incubation with tissue extract reduce or abolish the antithromboplastin activity of protamine plasmas as they do that of normal and hemophilic plasmas. Protamine also exaggerates the antithromboplastic and anticephalin activity of hemophilic plasmas but, as shown in Table I and observed repeatedly, not to as great a degree, comparatively speaking, as when the protamine is added to normal plasma. While the plasma clotting time of protaminized (0.04 g %) normal plasma is about 12 times that of intact plasma, that of hemophilic plasma with an equivalent protamine concentration is prolonged only a little over 3 times. The antithromboplastic activity developed in normal plasma by addition of protamine is apparently greater than that developed in hemophilic plasma by equivalent amounts of the same substance.

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[6] Mills, C. A., *Am. J. Physiol.*, 1926, **76**, 632.

[7] Eagle, H., *J. Gen. Physiol.*, 1935, **18**, 813.

[8] Chargaff, E., Bancroft, F. C., and Stanley-Brown, M., *J. Biol. Chem.*, 1936, **116**, 237.

[9] Howell, W. H., *Physiol. Rev.*, 1935, **15**, 435.

[10] Chargaff, E., and Ziff, M., *J. Biol. Chem.*, 1939, **131**, 25.

[11] Waldschmidt-Leitz, E., Stadler, P., and Steigerwaldt, F., *Z. f. phys. chem.*, 1929, **183**, 39.

It is plain, however, that the anticoagulating action of protamine is not solely due to its antithromboplastic properties. As Ferguson[12] has pointed out protamine has also antiprothrombic properties. The prolongation of the prothrombin time of the protaminized plasma (Table I) is probably due in part to this effect. Protamine is also known to precipitate fibrinogen to the extent of even "defibrinogenating" the blood and rendering it incoagulable.[13] 1.5 mg of protamine per cc of plasma precipitates approximately 80% of the total fibrinogen of that plasma.[13] By keeping the concentration of the protamine below 0.5 mg per cc of plasma the antithromboplastic effect is permitted to assert itself with a minimum precipitation of fibrinogen. Obviously, unlike the natural plasma antithromboplastin, protamine exerts its effects at several levels of the coagulation mechanism. Antithromboplastin is probably much simpler in action and composition than the protamines but may, like them, be strongly basic and thereby able to combine with cephalin, forming undissociable inert compounds. That this hypothesis may have some merit is indicated by the results of experiments, to be reported elsewhere, with anticoagulant basic dyes (toluidine blue, janus green) possessing antithromboplastic properties.

Summary. The plasma antithromboplastin seems to act on the cephalin moiety of the thromboplastic lipoprotein. Hemophilic plasma exerts a greater effect on cephalin than normal plasma. Normal plasma to which a certain amount of protamine has been added behaves towards cephalin and thromboplastin like hemophilic plasma.

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[12] Ferguson, J. H., *Am. J. Physiol.*, 1940, **130**, 759.

[13] Mylon, E., Winternitz, M. C., and de Suto Nagy, G. J., *J. Biol. Chem.*, 1942, **143**, 21.

14319

Growth of Rats on Synthetic Diets Containing Limiting Amounts of Thiamine.*

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Richardson and Hogan[1] reported that on a synthetic diet the 8 known vitamins were the only essential vitamins necessary for a rat to attain normal weight and maturity. Henderson *et al.*[2] and Unna *et al.*[3] also reported successful growth and reproduction on similar rations but found that when these diets were supplemented with whole liver or liver extracts greater growth responses were obtained than when the synthetic rations were fed alone.

Since optimum growth rarely results in assay experiments it has undoubtedly occurred to many workers that these simplified diets might be used for the determination of the individual vitamins in natural products. This procedure would greatly simplify some of the biological methods now in use. However one important question must be answered, namely, will a unit amount of a vitamin give the same response on these diets when fed as a free vitamin as when it is supplied in natural materials which carry many other factors?

We have attempted to answer this question in the case of thiamine and some of the results are presented in this paper.

Experimental. Several yeast and liver preparations were studied to determine which would produce the greatest growth responses when added to the synthetic ration with and without thiamine supplementation. Thirty-two male rats were divided into 2 groups. One group received ration 227 which consisted of casein, 18%; sucrose, 75%; salts IV, 4%; corn oil, 3%; pyridoxine, 200 γ; riboflavin, 300 γ; nicotinic acid, 250γ; pantothenic acid, 2 mg; and choline, 100 mg per 100 g of ration; the other group received ration 227 plus 150 γ of thiamine per 100 g of ration. All rats received one drop of halibut liver oil weekly. Yeast and liver preparations were fed to 2 rats of each group and were added to the ration at the expense of the sugar. The average weekly gains of these rats over a 5-week experimental period are shown in Table I.

The rats which received ration 227 plus 2% sulphited liver extract[4] (ration 228) plus 150 γ of thiamine per 100 g of ration grew better, 35.7 g per week, than did the rats on any of the other rations. The rats on ration 228 became polyneuritic at the same time as the controls on ration 227. This eliminated the possibility that the liver extract furnished thiamine to the ration.

To determine the differences in the rate of growth of animals on these rations with various levels of thiamine 8 groups of 3 rats each were placed on ration 227 and 228 supplemented with 0, 60, 80, 100, and 150 γ of thiamine per 100 g of ration. In every case the growth of the rats on ration 228 was superior to the growth on ration 227, Table II. The greatest difference in growth on the 2 rations appeared in rats receiving 80 γ of thiamine per 100 g of ration.

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We are indebted to Merck and Company, Rahway, N.J., for supplies of the crystalline vitamins; to Abbott Laboratories, North Chicago, Ill., for the halibut liver oil; and to Wilson Co., Chicago, Ill., for the liver extracts.

[1] Richardson, L. R., Hogan, A. G., Long, B., and Itschner, K. I., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 530.

[2] Henderson, L. M., McIntire, J. M., Waisman, H. A., and Elvehjem, C. A., *J. Nutrition*, 1942, **23**, 47.

[3] Unna, K., Richards, G. V., and Sampson, W. L., *J. Nutrition*, 1941, **22**, 553.

[4] Kline, O. L., Hall, W. L., and Morgan, J. F., *J. A. O. A. C.*, 1941, **24**, 147.

TABLE I.
Growth of Male Rats Receiving Ration 227 Supplemented with Various Liver and Yeast Preparations.

Supplement	Growth in g per week 0.0 γ thiamine	150 γ thiamine
None	polyneuritis 4 weeks	28.9
2% 1:20 sulphited liver extr.	’’ ’’ ’’	35.7
5% sulphited yeast	’’ ’’ ’’	32.1
2% butanol extr. of 1:20 liver extr.	’’ ’’ ’’	30.6
0.5% 1:20 liver extr.	’’ ’’ ’’	30.8
1% liver fraction B	’’ ’’ ’’	25.0
5% autoclaved yeast	’’ ’’ ’’	29.0
2% solubilized liver fraction L	’’ ’’ ’’	26.0

The sulphited liver extract was autoclaved for 3 hours at pH 5.7 to determine the stability of the active factors present. The preparation was fed at a level of 2% to a group of 3 rats receiving ration 227 plus 80 γ of thiamine per 100 g of ration. Since none of the activity was lost it was thought that p-aminobenzoic acid and inositol might be the active compounds. An experiment was devised to study the effect of these substances: 4 groups of 3 male rats each were fed the following rations, 227 plus 80 γ of thiamine; 227 plus 80 γ of thiamine and 30 mg of p-aminobenzoic acid; 227 plus 80 γ of thiamine and 100 mg of inositol; and 227 plus 80 γ of thiamine, 30 mg of p-aminobenzoic acid, and 100 mg of inositol per 100 g of ration. The results of these experiments are shown in Table III.

TABLE II.
Growth of Male Rats Receiving Rations 227 and 228 Supplemented with Various Levels of Thiamine.

γ of thiamine per 100 g of ration	Growth in g per week Ration 227	Ration 228
0.0	polyneuritis 4 wks	polyneuritis 4 wks
60.0	4.0 g	11.0 g
80.0	7.9	20.0
100.0	18.0	27.0
150.0	27.0	33.0

TABLE III.
Growth of Rats Receiving Rations 227 Supplemented with Inositol, p-Aminobenzoic Acid and Liver Extract.

Supplement per 100 g of ration	No. rats	Growth g/wk
80 γ thiamine	9	12.8
2% autoclaved sulphited 1:20 liver extr. + 80 γ thiamine	3	20.0
30 mg p-aminobenzoic acid + 80 γ thiamine	3	12.7
100 mg inositol + 80γ thiamine	3	14.2
30 mg p-aminobenzoic acid, 100 mg inositol and 80 γ thiamine	9	16.3

Rats which received the p-aminobenzoic acid or inositol respectively failed to show growth responses over that obtained on ration 227. However when the two compounds were fed together a definite growth response was obtained. Considerable variation was observed in these studies but in 3 individual experiments rats receiving both p-aminobenzoic acid and inositol showed a definite growth increase over those receiving ration 227.

Discussion. Considerably better growth was obtained when sulphited liver extract was added to the synthetic ration even when suboptimal levels of thiamine were added. The greatest growth differences were observed when 80 γ of thiamine were fed per 100 g of ration. It is thus impossible to use a synthetic ration for assay purposes since the natural materials to be studied may cause a growth stimulation similar to that obtained with the liver preparation.

A comparison of the growth curves of rats receiving ration 227 and 228 to which 0, 80, and 150 γ of thiamine were added per 100 g of ration is interesting (Fig. 1). The growth of rats on the two rations receiving 150 γ of thiamine was very similar, especially after 6 or 7 weeks, although the rats receiving the liver extract grew faster during the first 5 weeks on experiment. The rats on ration 228 which received 80 γ of thiamine grew considerably faster than those on ration 227, although animals on both rations plateaued at the same time, approximately 4 weeks. The animals on the basal rations became polyneuritic at the same time. These facts eliminate the possibility of a thiamine-sparing action.

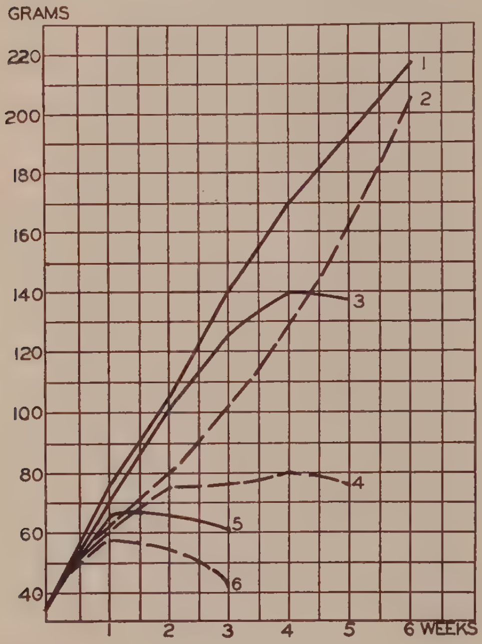

Fig. 1.

Growth curves of rats receiving: 1, 228 plus 150 γ thiamine; 2, 227 plus 150 γ thiamine; 3, 228 plus 80 γ of thiamine; 4, 227 plus 80 γ of thiamine; 5, 228 basal; and 6, 227 basal.

The increased growth produced by the liver preparation may be explained on the basis of one of two mechanisms. Either the extract furnished a growth-stimulating substance which has a greater effect when suboptimal levels of thiamine are fed, or it furnished a condition for an intestinal flora which synthesizes growth-stimulating factors that are produced to some extent when the rat is receiving a normal diet. This seems possible since similar effects have been noted on suboptimal pantothenic acid rations for growth,[3] and for protection against grey hair.[5]

The p-aminobenzoic acid and inositol probably exert the same effect that the liver extract does. Martin[6] has suggested that p-aminobenzoic acid alters the intestinal flora in achromotrichia to affect the synthesis of "folic acid." Pavcek[7] reported that inositol showed a growth-promoting activity when fed to pyridoxine-deficient animals. Inositol has recently been shown to play a role in lactation of the rat,[8] and Engel[9] states that inositol is a necessary constituent in highly purified diets for the rat.

Ration 228 has been used as a basal ration for the determination of thiamine in crude materials. Both curative[10] and growth technics have been used. Results obtained by the growth method and the thiochrome assay method show good correlation but studies involving a much larger number of rats are necessary before a more exact comparison of the two methods can be made.

Summary. Superior growth was obtained when synthetic diets containing various levels of thiamine were supplemented with sulphited liver extract. The addition of p-aminobenzoic acid and inositol to the synthetic ration also increased the rate of growth of rats receiving suboptimal levels of thiamine.

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[5] Frost, D. V., Moore, R. C., and Dann, F. P., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 507.

[6] Martin, G. J., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 353.

[7] Pavcek, P. L., and Baum, H. M., *Science*, 1941, **93**, 502.

[8] Climenko, D. R., and McChesney, E. W., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 157.

[9] Engel, R. W., *J. Nutrition*, 1942, **24**, 175.

[10] Waisman, H. A., and Henderson, L. M., unpublished data.

14320

Effect of Carbon Arc Irradiation and Adrenal Cortical Preparations on Capillary Permeability.*

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In studies concerning the cause of the reduction in blood pressure following carbon arc irradiation, it was of interest to obtain an estimate of the extent and degree of changes in capillary permeability produced by the irradiation. For this purpose Menken's trypan blue test[1] was used on rabbits.

The animals were divided into 2 groups of 10 each. Group I served as normal controls; Group II were irradiated over the abdominal area. The abdomen of all the rabbits was shaved at approximately the same time. One hour later Group II was exposed to the carbon arc lamp for 30 to 40 minutes at a distance of one meter. Four hours later all animals received intravenously 8 to 10 cc of 1% solution of trypan blue in normal saline. Two hours after injecting the dye the animals were carefully examined over the irradiated area of the abdomen, then killed, and the viscera compared with reference to the degree of staining by the dye.

The results showed striking differences. The skin and abdominal viscera of control animals presented a blue tinged appearance with the exception of the liver which appeared normal in color. The irradiated animals showed an extreme blue coloration of the skin and viscera many times more marked than that of the controls. The livers of these animals also showed coloration in contrast to the normally appearing livers of Group I. There was usually a collection of blue stained fluid (about 5 cc) in the abdominal cavity. Photographs in color were taken of a typical series to provide a permanent and objective record of the differences.

It would seem that irradiation of the rabbit with the dosage used in these experiments produces changes in the vascular system of the skin and abdominal organs which allow an increased amount of injected dye to escape from the blood stream and infiltrate the tissues.

Since the blood pressure reductions by the carbon arc can be prevented in the dog by administration of adrenal cortical substances[2] it seemed desirable to determine whether these substances also prevent the increase in capillary permeability. A third group of 10 animals was added to the experiments. These were first treated with 10 mg of desoxycorticosterone acetate or 5 cc of cortin intramuscularly immediately before, during, or immediately after the irradiation. The dye injection then followed in the same manner as for the animals of Group II. Upon examination after injection of trypan blue, the tissues of these animals receiving cortical substances before irradiation showed changes indistinguishable from the control group. Apparently the effects of irradiation upon the capillaries of tissues, both immediate and distant to the exposed area, are prevented or markedly diminished by previous administration of adrenal cortical extract or desoxycorticosterone acetate. These results indicate that the adrenal cortical hormones are capable, under certain conditions, of preventing the increased permeability occurring in the minute vessels by the action of substances released from tissue undergoing inflammatory changes.

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* Aided by a grant from the Committee on Scientific Research of the American Medical Association to Professor Henry Laurens. The National Carbon Co. loaned the lamps and furnished the carbons. The Ciba Pharmaceutical Products, Inc., and Roche-Organon, Inc., generously contributed the desoxycorticosterone acetate. Roche-Organon, Inc., and the Upjohn Co. gave ample supplies of adrenal cortical extract.

[1] Menkin, V., *Dynamics of Inflammation*, Macmillan Company, New York, 1940, pp. 18-23.

[2] Laurens, H., and Graham, J. S., *Med. Rec.*, 1941, **154**, 146.

This is in agreement with the findings of Menken[3] who studied the effect of the adrenal cortical substances on the accumulation of trypan blue in local lesions produced by the injection of leukotoxine or inflammatory exudate.

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[3] Menken, V., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 39.

14321 P

Factors Controlling Lens Regeneration from the Dorsal Iris in the Adult *Triturus viridescens* Eye.*

L. S. Stone.

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When the adult eye of *Triturus viridescens* is transplanted[1,2] or its blood supply is temporarily lost[3] the lens degenerates along with most of the retina. In these cases a new lens is developed from the dorsal pupillary margin of the iris such as follows simple lensectomy.[3,4] This phenomenon of lens replacement is largely, if not exclusively, confined in the vertebrates to the Triturus group of salamanders[5,6,7] where the lens appears to be less resistant to injury than in other forms.[5,8] An extensive study is being made by the author in search for the factors which govern this phenomenon. Some of the findings can be summarized at this time.

Slight injuries to the fully developed lens in the adult eye of *Triturus viridescens* cause it to degenerate. Injuries to the dorsal iris produced by mechanical stretching and incisions radiating from the pupillary margin neither prevent nor favor lens regeneration. Such injuries heal readily. Only the presence of the normal lens prevents a new one from forming. This was shown by means of a successful pipette method of removing and reimplanting the lens in the adult eye without injury.

If a piece of the dorsal iris from a normal eye or from one lensectomized 6 or 7 days earlier is transplanted to a freshly lensectomized host eye, a new lens will develop from the graft. If, however, such grafts are placed in an eye possessing a normal lens, the latter sharply inhibits lens regeneration in all cases. Even in those grafts where the implanted iris was allowed to initiate early preparation[7] for lens regeneration through delay before transplantation, only a rare case showed at best a feeble, short-lived attempt to progress further before it was permanently arrested. The presence of the normal lens of the host eye apparently exerts an early effect upon the lens-forming capacity of the implanted iris.

Varying the time from the day of operation to 25 days after lensectomy in both host and donor eyes, combinations were obtained which tested the effects of older and younger regenerating lenses on each other in the same environment. The experiments proved that the presence of a regenerating lens from an iris up to 25 days after lensectomy does not inhibit nor prevent the regeneration of a lens from other dorsal iris tissue in its environment. Under some circumstances as many

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* Aided by grants from the John and Mary R. Markle Foundation and the Fluid Research Fund of Yale University.

[1] Stone, L. S., and Zaur, I. S., *J. Exp. Zool.*, 1940, **85**, 243.

[2] Stone, L. S., and Farthing, T. E., *J. Exp. Zool.*, 1942, **91**, 265.

[3] Stone, L. S., and Chace, R. R., *Anat. Rec.*, 1941, **79**, 333.

[4] Ogawa, C., *J. Exp. Zool.*, 1921, **33**, 395.

[5] Stone, L. S., and Sapir, P., *J. Exp. Zool.*, 1940, **85**, 71.

[6] Stone, L. S., and Dinnean, F. L., *J. Exp. Zool.* 1940, **83**, 95.

[7] Dinnean, F. L., *J. Exp. Zool.*, 1942, **90**, 461.

[8] Stone, L. S., and Cole, C. H., *Yale J. Biol. and Med.*, 1943, **15**, 735.

as 3 lenses may grow in the same environment. However, between 25 and 30 days after lensectomy the regenerating lens demonstrates clearly that at this time it becomes linked with a factor, as in the case of a normal fully grown lens, which acts as an inhibitor for lens regeneration from the iris within the eye. This is at a time when the newly forming lens detaches itself from its source of origin, and probably marks a definite period in its differentiation. Both implantations of the detached lens and implantations of various types of dorsal iris emphasize this point.

The results of the experiments carried out within the eye environment seem to be significant. So far it has been found that the dorsal iris grafts of the adult eye of this species only rarely made a feeble but aborted attempt to regenerate a lens when placed outside of the eye environment such as under the skin or within the body cavity. However, this inhibiting effect is possibly of a different nature than that exerted by the lens itself.

14322

Concentration and Preservation of Crude Penicillin.

Tsun T'ung. (Introduced by Martin Frobisher, Jr.)

From the Department of Bacteriology, School of Hygiene and Public Health, Johns Hopkins University.

Rapid advances in methods for the purification of penicillin seem to have rendered the small-scale laboratory production of the crude material undesirable. However, since the processing of pure penicillin is complicated and the yield is very small, general clinical trials and intensive laboratory investigations of this substance will not be possible for some time to come. Therefore, the crude penicillin is still useful, provided its bulk can be reduced without loss of potency. The present communication presents a simple and inexpensive procedure for concentrating and preserving crude penicillin for practical use.

The Fleming strain of *Penicillium notatum*[1] was grown on modified Czapek-Dok medium[2] contained in Blake bottles. In order to minimize precipitation of the constituents of the medium, brown sugar and ferrous sulfate were separately sterilized by autoclaving and filtration through Seitz pad, respectively, and were then added to the basic medium.

After one week of incubation at room temperature the penicillin titer of each bottle was determined by the serial dilution method. Meat infusion broth with 1% neopeptone at pH 7.4 was used as diluent. A strain of Lancefield Group A beta hemolytic streptococci was chosen at random as the test organism. Each tube of diluted penicillin was seeded with a drop of an 18-hour broth culture containing about 30 million organisms.

Cultures having effective titers in a dilution of 320 or over were pooled. The pH was adjusted to 6 and 500 ml were poured into a 700 ml distilling flask through cheesecloth. The flask was partly immersed in a water bath the temperature of which was maintained at 60°C. The substrate containing penicillin was evaporated under reduced pressure. The process is illustrated by the accompanying diagram (Fig. 1). A vacuum of about 0.001 mm of mercury was created by a running water pump or the Cenco-Hyvac pump. In 3 hours, the volume of 500 ml could be reduced to 20 ml. The pH of the concentrated stuff was readjusted to 6 and it was sterilized by filtration. While the volume of the crude penicillin was reduced, the titer of the active principle increased manyfold.

The concentrated penicillin was kept in celluloid tubes. Each lot was divided into two portions; one was placed in a dry-ice cabinet at a temperature of –76°C[3] and the other in an ordinary ice box at 5°C. Determinations of the titer of the preserved material against the same organism, using the same medium as previously, were made from time to time. Titrations of potency were performed on the first three consecutive days of storage to insure the accuracy of the initial titer. The keeping qualities of 3 lots of crude penicillin of different titers at the two temperatures are presented in Fig. 2.

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[1] Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 226.

[2] Hobby, Gladys, and Myer, Karl, Proc. Soc. Exp. Biol. and Med., 1942, **50**, 227.

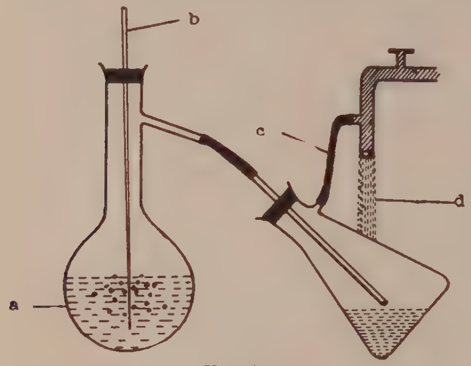

FIG. 1.

Partial Vacuum Concentration Apparatus.
a. Crude culture of *Penicillium notatum.*
b. Tube with capillary tip to produce ebullition.
c. Tube to vacuum pump.
d. Stream of cool water to condense vapor.

At a temperature of –76°C the potency of crude penicillin could be maintained for about one month without marked deterioration. After one to 2 months titers dropped about 50% but remained stable for at least 3 to 5 months thereafter. The same substance kept at 5°C lost its potency rapidly and almost entirely toward the end of 3 months. This seems not to be in accordance with the observations made by Abraham *et al.*[4] and Clutterbuck *et al.*[5] The latter could maintain the potency of a crude penicillin filtrate for 3 months at 0°C and the former stated that in aqueous solution the purified penicillin at 2°C is stable for several months. Probably constituents of the medium and other impurities after being concentrated are responsible for this difference. As facilities for maintaining these temperatures are easily available in ordinary laboratories, the keeping quality of the concentrated crude penicillin at other temperatures was not attempted.

The importance of the adjustment of pH of crude chrysogenin to between 5 and 6 before concentration to render the substance stable during evaporation has been stressed by Clutterbuck.[5] The same pH range is essential for the concentration of penicillin. The pH also plays important roles in the process of concentration and the determination of the titer of penicillin. When the pH was above 6 the culture fluid, when heated, foamed so much that the process of concentration had to be discontinued at once. In titrating a given lot of penicillin the use of a diluent of pH 7.2 may result in a titer twice as high as that obtained with a diluent having a pH 7.6.

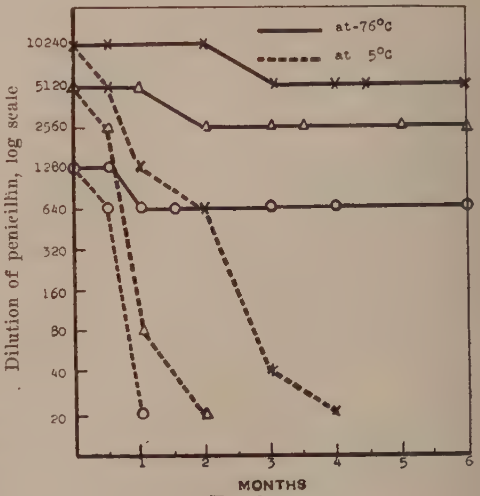

FIG. 2.

Keeping quality of concentrated penicillin at different temperatures.

As is true of spirochaetes and viruses[6] frequent freezing and thawing does not have any appreciable deleterious effect on the preserved crude penicillin. This property permits a convenient and repeated use of the preserved stuff with maintenance of potency for at least 6 months.

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[3] Horsfall, Frank K., Jr., *J. Bact.*, 1940, **40**, 559.

[4] Abraham, E. P., and Chain, E., *Brit. J. Exp. Path.*, 1942, **23**, 103.

[5] Clutterbuck, P. W., Lovell, Reginald, and Raistrick, Harold, *Biochem. J.*, 1932, **26**, 1907.

[6] Turner, Thomas B., *J. Exp. Med.*, 1938, **67**, 61.

Summary. The volume of crude penicillin can be greatly reduced with considerable increase in potency of the active principle by a process of low temperature-partial vacuum concentration. After an initial drop the titer of the concentrated material kept in a dry-ice cabinet is maintained for as long as 6 months.

14323

Synergistic Action of Thiourea and Guanidine with Sulfathiazole.

S. W. LEE, JEANNE A. EPSTEIN, AND E. J. FOLEY.* (Introduced by Marion B. Sulzberger.)

From the Wallace Laboratories, Inc., New Brunswick, N.J.

An advantageous co-action between sulfonamides and urea has been found to exist, both in wounds,[1] and *in vitro*.[2] The conclusion reached by Tsuchiya *et al.* in the latter case have been contested by Kirby.[3] In this paper we have confirmed the finding of Tsuchiya *et al.*, and have extended the observations to include a study of thiourea and iminourea (guanidine). The structural similarity of these two compounds to urea is obvious from the formulæ:

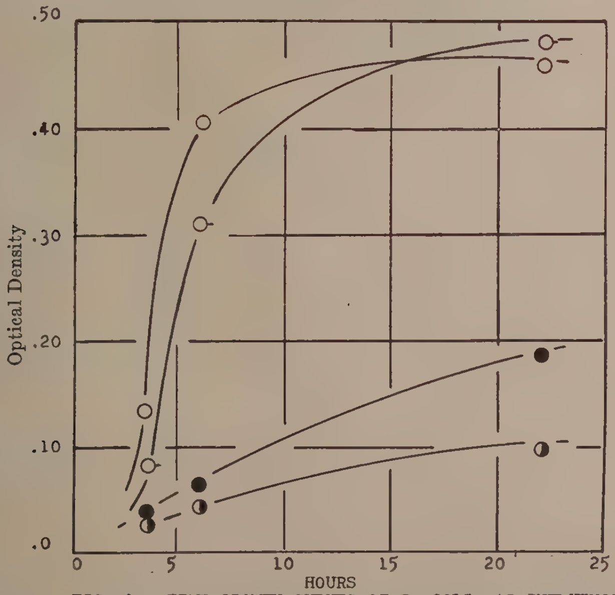

FIG. 1. TIME-GROWTH CURVES OF E. COLI, AS INFLUENCED BY UREA, SODIUM SULFADIAZINE AND THEIR COMBINATION.

○ = Control.
○- = Urea, 1.6% or 0.28 M.
● = Sodium Sulfadiazine, 1 mg. %.
◑ = Urea, 1.6% or 0.28 M, and sodium sulfadiazine, 1 mg.%.

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* The authors wish to acknowledge the assistance of Miss Isabel Perrine.

[1] Holder, H. G., and MacKay, E. M., *Military Surg.*, 1942, **90**, 509.

[2] Tsuchiya, H. M., Tenenberg, D. J., Clark, W. G., and Strakosch, E. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 247. See also previous papers.

[3] Kirby, W. M. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 109.

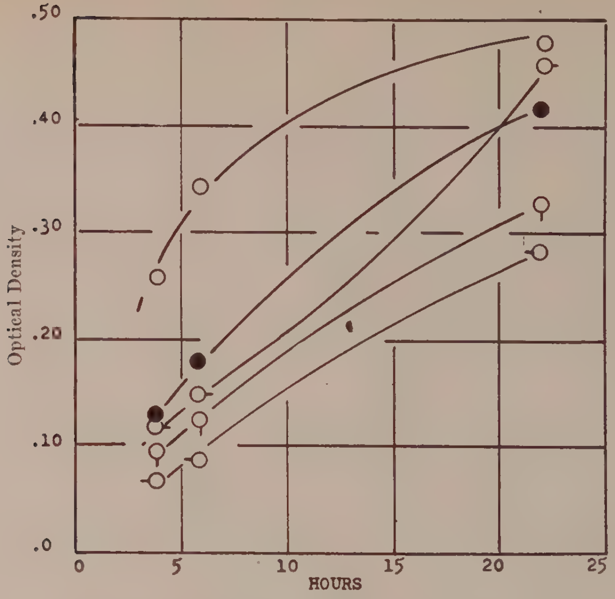
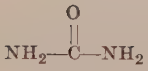
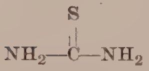


FIG. 2. TIME-GROWTH CURVES OF E. COLI, AS INFLUENCED BY SULFATHIAZOLE ALONE AND COMBINED WITH UREA, THIOUREA OR GUANIDINE.

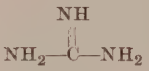
○ = Control.
● = Sulfathiazole, 10^{-5}M
○- = Sulfathiazole, 10^{-5}M, and urea, 0.066 M.
♀ = Sulfathiazole, 10^{-5}M, and thiourea, 0.066 M.
-○ = Sulfathiazole, 10^{-5}M, and guanidine, 0.066 M.


$NH_2-\overset{\overset{O}{\|}}{C}-NH_2$

Urea


$NH_2-\overset{\overset{S}{\|}}{C}-NH_2$

Thiourea


$NH_2-\overset{\overset{NH}{\|}}{C}-NH_2$

Guanidine

Thiourea and guanidine have been found to be much more active than urea, in the above sense.

The early work of Tsuchiya *et al.*[2] has been repeated in a quantitative manner. Fig. 1 clearly shows these results. The conditions for these experiments were identical to those of Tsuchiya *et al.*, except for the initial population of *E. coli,* which was about 6 million organisms per ml. The stimulatory effect of urea (after 24 hours) at the concentration used (1.6%, 0.28 M), as well as its synergistic action with sodium sulfadiazine, is shown. The bacterial growth was determined turbidimetrically, and the density readings plotted in Fig. 1 are taken directly from the drum of a Coleman Universal Spectrophotometer. With this organism, the population is roughly linearly related to the optical density. As determined by plating, the bacterial count per ml corresponding to a density of 0.46 is 6.5 x 10^8. Using the exact plating method of Tsuchiya *et al.*, the counts obtained were well in line with the related density readings.

It is seen from Fig. 1 that urea alone exerts a slight bacteriostatic effect in the early part of the growth curve, in essential agreement with the work of Kirby. However, the magnitude and even the presence of this growth diminution is dependent on the inoculum size. This dependence is discussed in the subsequent paper. Otherwise, the results are similar to those of Tsuchiya *et al.*, but are here given quantitatively.

As was the case with urea, preliminary experiments were carried out to determine the lowest concentration of thiourea and guanidine which did not adversely affect the growth rate of *E. coli,* in a synthetic medium consisting of glucose and various salts (SG).[4] A concentration of 0.066 M was chosen, and equimolar solutions of urea, thiourea, and guanidine (in the form of its sulfate) were made up in sterile SG medium. One ml of sulfathiazole solution (10^{-3} M) was added to 99 ml portions of the above solutions for the mixed drug solutions. These, together with SG medium (control) and 10^{-5} M sulfathiazole in SG medium, were added in 10 ml amounts to sterile tubes. One-half ml of a 1:2.5 dilution of an overnight culture of *E. coli* was added to each tube, giving an initial population of about 1.1 x 10^6 organisms per ml. The suspensions were incubated at 37°, and their densities determined at various times. The result of this experiment is shown in Fig. 2, each point representing an average of 4 or 5 determinations.

It can be seen from Fig. 2 that, over the entire period of 24 hours, the order of increasing synergistic action is urea, thiourea, and guanidine. At 24 hours, the time chosen by Tsuchiya *et al.*,[2] the picture is more favorable for the latter two compounds. The urea, in the presence of the greater number of organisms (which is essentially a dilution from the standpoint of a single enzyme molecule) shows a growth stimulating effect. The presence of thiourea (0.066 M) brings about a 22%, and guanidine a 34% growth decrease, in addition to that caused by the sulfathiazole (10^{-5} M), which was about 13%. The figures on thiourea and guanidine are on the basis of the growth of the organism in the presence of sulfathiazole (10^{-5} M), which is the related control.

Summary. Guanidine and thiourea, in this order, have been found to be much more active than urea (all at the same molar concentration), when used jointly with sulfathiazole in inhibiting the growth of *E. coli* on a simple medium. The action is a synergistic one.

The synergistic action of urea with sodium sulfadiazine on *E. coli* has been confirmed, using the conditions defined by Strakosch and associates.

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[4] Kohn, H. I., and Harris, J. S., *J. Pharm. and Exp. Therap.*, 1943, **77**, 1.

14324

Synergism of Urea and Sulfathiazole on *E. coli*. Effect of Inoculum Size.

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From the Wallace Laboratories, Inc., New Brunswick, N.J.

In a previous paper (see companion article) the varying opinions of Tsuchiya *et al.*[1] and Kirby[2] concerning the nature of the action of urea with the sulfonamides upon *E. coli* has been mentioned. In conjunction with other problems, a few experiments have been carried out in this laboratory which are considered to bear on this problem.

It should first be pointed out that the experiments of Kirby[2] were not carried out exactly as were those of Tsuchiya *et al.*, and because of these variations the results should probably not have been contrasted. The obvious points of difference in the two series of experiments were media and inoculum size. The medium of Kirby contained sugar, salts and casamino acids, Difco, whereas that of Tsuchiya *et al.* consisted only of salts and glucose, similar in nature to that which has been used in experiments reported here (medium SG[3]).

The much greater departure in conditions, it is felt, concerns the inoculum size. Tsuchiya *et al.* worked with the large initial population of about 800,000 organisms per ml. Kirby

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[1] Tsuchiya, H. M., Tenenberg, P. J., Strakosch, E. A., and Clark, W. G., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 245. (See also previous papers.)

[2] Kirby, W. M. M., Proc. Soc. Exp. Biol. and Med., 1943, **53**, 109.

[3] Kohn, H. I., and Harris, J. S., *J. Pharm. and Exp. Therap.*, 1943, **77**, 1.

TABLE I.
Sizes of Inocula of *E. coli* and the Bacteriostatic Action of Urea, Sulfathiazole and Their Combination.

	Optical densities									
Initial Population	6×10^7 org/ml		6.5×10^6 org/ml		1.3×10^6 org/ml		6.5×10^5 org/ml		6.5×10^4 org/ml	
Hrs after inoculation	5	21	5	21	5	21	5	21	5	21
Substances added to medium										
Control, no drug	.28	.32	.18	.36	.06	.20	.03	.19	0	.14
Sulfathiazole (STA) 10^{-5} M	.30	.35	.05	.23	.01	.22	—	.16	0	.01
Urea 0.2%	.28	.28	.17	.26	.05	.28	.02	.25	0	.24
" 0.5%	.34	.29	.16	.32	.05	.26	.02	.28	0	.26
" 1.0%	.29	.36	.11	.40	.02	.33	0	.33	0	.28
" 2.0%	.28	.35	.09	.24	.02	.28	0	.25	0	.26
(STA) 10^{-5} M + Urea .2%	.29	.34	.05	.28	0	.22	0	.15	0	0
" " " " " .5%	.30	.33	.05	.23	0	.22	0	.13	0	0
" " " " " 1.%	.27	.32	.05	—	0	.09	0	.02	0	0
" " " " " 2.%	.21	.26	.022	.12	0	.03	0	.02	0	0

does not mention the inoculum size used in his work, but the nature of his graphs indicates it to have been rather small.

The effect of the size of the inoculum on the results obtained in testing bacteriostatic substances is well known.[4] In general, small inocula are considered to give unsatisfactory or misleading results. The work on this phase of the problem of urea-sulfonamide synergism is given in this report.

Method. The methods used were essentially those previously reported.[5] Growth curves of *E. coli* were measured turbidimetrically at critical times, 5 and 21 hours, using a Coleman universal spectrophotometer with the wage-length dial set at 600 mμ. The optical densities are given as such, a value of 0.30 corresponding to a population of 6.5×10^8 organisms per ml. The population, as determined by plate count, is roughly linearly related to the density.

In the experiments, 10 ml of medium SG containing the desired concentration of the drugs was added to a series of sterile tubes. The varying inocula were added to these tubes. The culture (18-hour) used for inoculation contained 6.5×10^8 organisms per ml, and volumes of from 1 ml of this culture to 0.10 ml of its 1:100 dilution were added to the tubes, giving the initial population indicated in Table I. Each determination was done in triplicate. The tubes were incubated at 37°C.

Results. Table I indicates the marked effect of inoculum size on the results obtained. With very large inocula the growth stimulatory effect of urea alone is manifest (*cf.* Tsuchiya *et al.*), while with the small size it becomes bacteriostatic (*cf.* Kirby). The effect of sulfathiazole (10^{-5} M) is comparable. The combination of these two always has a synergistic effect—slight with large inocula, but very pronounced with the smaller. The 21-hour data from initial population of 1.3×10^6 organisms per ml corresponds well to that of Tsuchiya *et al.* Though the urea alone acts to retard growth during the first few hours, its action with sulfathiazole must still be considered synergistic.

It has been shown that part of the divergent opinions concerning the conjoint action of urea must result from the use of different experimental conditions.

Summary. When a salt-glucose medium is used, the size of the inoculum of *E. coli* has been shown to play the dominant part in studies concerning the effect on growth rates of urea, sulfathiazole, and their combinations. The action of these two alone has been shown to vary from stimulatory, with the very large inocula, to markedly bacteriostatic with the smaller. When used together, sulfathiazole and urea (in several concentrations) act synergisticly with all inocula sizes, the combined effect being much greater with the smaller inocula.

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[4] MacLeod, C. M., and Mirick, G. S., *J. Bact.*, 1942, **44**, 277.

[5] Lee, S. W., and Foley, E. J., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 243.

14325

Limited Effects of Certain Steroid Hormones on Mammary Glands of Hypophysectomized Rats.*

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From the College of Veterinary Medicine and Department of Zoology, Cornell University, Ithaca, New York.

The controversial nature of the reports on mammary growth of hypophysectomized mammals treated with steroid hormones has apparently been caused in part by species differences and by different physiological conditions of the animals subjected to treatment. Certain steroid hormones have induced mammary growth in hypophysectomized mice[1] and rabbits,[2] and under particular circumstances, similar results were reported in hypophysectomized rats.[3] While the mammary glands of hypophysectomized rats gave specific growth effects with testosterone regardless of age or when treatment was begun following ablation of the pituitary, they did not respond to estrogen unless the rats were immature and under 70 grams body weight, and the injections were begun immediately following the operation.

In view of the reported favorable mammary growth in hypophysectomized mice and rabbits with progesterone and the negative effects of this hormone in hypophysectomized rats,[4,5] an investigation of the influences of this hormone alone or in combination with estrogen was undertaken. Studies were also made on the effects of desoxycorticosterone acetate under conditions which permit estrogen to stimulate mammary growth in hypophysectomized rats.

Methods. Male and female rats of the Long-Evans strain, ranging from 26-38 days of age and weighing from 50 to 98 g at the time of operation were used. As far as possible, comparisons were made between glands of animals within the same litter. An adequate diet supplemented with a 20% glucose solution was fed *ad libitum.*

Experiments were designed to include both immediate and postponed treatment following hypophysectomy. The total amounts of the hormones injected for a 10-day period were progesterone (Lutocylin), 10-30 mg; α-estradiol dipropionate (Di-Ovocylin), 50 γ; desoxycorticosterone acetate (Percorten), 7.50-10.0 mg.[†] At autopsy, the right abdominal mammary gland was removed and stained *in toto* with Mayer's hemalum. Observations were made on adrenal, testis, and body weights, and all operations were verified as complete by examining the sellæ turcicæ histologically. Sixty-one completely operated animals were used in these experiments.

Results. Progesterone was observed to stimulate growth of the end buds and to thicken slightly the ducts of the mammary glands in 3 of 5 hypophysectomized rats when dosages of 30 mg in 10 days were injected, beginning immediately after the operation. In 2 of 3 rats receiving 15 mg dosages of progesterone under the same conditions, only a slight enlargement of the ducts could be observed and only when compared with their littermate controls. If treatment was postponed for 4-8 days after hypophysectomy, the mammary glands of 2 animals receiving 30 mg of hormone could not be differentiated from the controls.

When progesterone was given in combination with a constant dose of estrogen, the amount of new mammary growth obtained in

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* Aided by a grant from the Sackett Fund of the Trustee-Faculty Committee on Research.

[1] Gardner, W. U., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 835.

[2] Asdell, S. A., and Seidenstein, H. R., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 931.

[3] Leonard, S. L., *Endocrinology,* 1943, **30**, 229.

[4] Gomez, E. T., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.,* 1937, 259.

[5] Selye, H., *Anat. Rec.,* 1940, **78**, 253.

[†] Dr. E. Oppenheimer of the Ciba Pharmaceutical Products, Inc., Summit, N.J., kindly furnished these hormones.

rats treated immediately after hypophysectomy varied with the dosage of progesterone. A combination of 1 mg of progesterone plus 5 γ of estrogen per day for 10 days resulted in mammary growth superior to that in rats receiving estrogen alone in only 1 of 3 rats. When 2 or 3 mg of progesterone were given with the same amount of estrogen, in all of 7 cases, the mammary growth was superior to that obtained with estrogen alone. This was characterized by more numerous stimulated end buds, increased lateral buds and branches, and decidedly thickened ducts, in contrast to fewer end buds and practically no duct enlargement when estrogen alone was given. The stimulated glands showed a summation of the effects of the individual hormones.

If treatment with the combined hormones was not begun immediately after hypophysectomy, no growth was obtained. Six rats hypophysectomized for 4-8 days and receiving 3 mg progesterone plus 5 γ of estrogen daily for 10 days, failed to show any evidence of growth. In no way could they be distinguished from control glands receiving only estrogen or from those of uninjected controls.

In the next experiment, desoxycorticosterone acetate was injected in daily doses of .75-1.0 mg for 10 days into 5 immature hypophysectomized rats starting immediately after the operation. These rats were of such age and weight as to be sensitive to injections of estrogen or progesterone under the conditions of the experiment. This hormone was without effect, for the mammary glands of the injected rats could not be distinguished from those of the uninjected controls.

The androgenic action of progesterone as reported by Nelson[6] was also observed in the rats receiving immediate treatment in that the testes of the hypophysectomized injected animals were 250-300% heavier than those of the untreated hypophysectomized controls. No effect was noted on the ovarian or adrenal weights.

Discussion. Progesterone, like estrogen, was found capable of inducing some mammary growth in immature hypophysectomized rats in a 10-day period, provided the dosage was adequate and was begun immediately after the operation. The type of growth, on the whole, resembled that obtained with estrogen in that end buds were stimulated, but in addition, the thickening of the duct system was characteristic of progesterone action. If the treatment was postponed following the operation, no growth was observed.

The combined estrogen and progesterone injections induced greater growth than either hormone alone in the animals receiving immediate treatment, but it should be emphasized that under no circumstances did the gland development approach that found in a normal animal. The effects of these hormone injections became significant only when the glands of treated animals were compared with those of untreated controls where, in the latter group, nothing but naked ducts were found. When treatment was postponed, the combined hormones were unable to stimulate growth.

Desoxycorticosterone acetate, which is capable of stimulating mammary growth in normal mice[7] or rats,[8] or in hypophysectomized mice,[1] was found inactive in hypophysectomized rats. The mammary glands of the rats selected for these experiments were capable of responding to estrogen, yet this cortical hormone was ineffective. Previously, it was reported that older hypophysectomized rats would not respond to desoxycorticosterone.[8]

Species differences between hypophysectomized rats and mice in their responses to estrogen[3] also occur for progesterone. Gardner[1] reported that estrogen plus progesterone or progesterone alone, was capable of stimulating the hypophysectomized mouse mammary gland any time from 1-89 days after the operation. In the rat, the conditions seem to be limited in which these hormones will stimulate the glands.

In the normal or castrate rat with pituitary intact, it had previously been found[9,10] that total doses of 16-48 mg of progesterone with or without estrogen over periods of 8-20 days were ineffective in stimulating mammary development above that of estrogen alone. Inasmuch as the growth obtained with similar doses in the present work with hypophysectomized rats was relatively minimal compared to normal growth, possibly any effect that might have been present in the normal rat would have been masked by the greater overall development of the normal gland. That this is likely is suggested by the fact that doses of 150 mg of progesterone over 10 days in spayed rats, or 225 mg over 15 days in rats spayed 9 days before injections, produced distinct mammary development with lobule formation.[11]

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[6] Nelson, W. O., *Anat. Rec.*, 1936, **67**, 110 (Suppl.).

[7] Van Heuverswyn, J., Folley, S. J., and Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 389.

[8] Leonard, S. L., and Reece, R. P., *Endocrinology*, 1942, **30**, 42.

Other workers have reported negative effects of progesterone on hypophysectomized rat mammary glands.[4,5] In the latter work a total amount of 100 mg of progesterone over a 10-day period or 200 mg over a 20-day period was used without securing growth. As the weights of the rats employed were somewhat heavier than those used in the experiments reported here, it would seem that the conditions necessary for new growth with progesterone are similar to those required for estrogen stimulation.

Progesterone exhibited androgenic activity in that it prevented the normal rate of atrophy of the testes following hypophysectomy, an action similar to that of testosterone. Although testosterone[3] and progesterone were found capable of increasing the thickness of the mammary ducts, testosterone was found to be more potent. A dosage of 6.25 mg of testosterone gave better results than 30 mg of progesterone in this respect.

These studies, while indicating that the hypophysectomized rat mammary gland may be stimulated by certain steroids, emphasize the necessity of adequate pituitary hormones to produce a fully developed mammary gland.

Summary. Hypophysectomized rats were treated immediately or several days after the operation with progesterone alone or in combination with estrogen and with desoxycorticosterone acetate. The mammary glands of these animals were stimulated with progesterone and estrogen alone or in combination only when treatment was begun immediately. The effects of the combined hormones revealed a summation of the individual responses. Desoxycorticosterone was ineffective.

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[9] Selye, H., Browne, J. S. L., and Collip, J. B., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 472.

[10] Astwood, E. B., Geschichter, C. F., and Rausch, E. O., *Am. J. Anat.*, 1937, **61**, 373.

[11] Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 343.

14326 P

Local Maintenance of Spermatogenesis in Hypophysectomized Rats with Low Dosages of Testosterone from Intratesticular Pellets.*

SAMUEL DVOSKIN. (Introduced by P. E. Smith.)

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The maintenance of spermatogenesis in hypophysectomized rats by androgen administration, first reported by Walsh *et al.*,[1] has been repeatedly confirmed. Reinitiation of spermatogenesis by this treatment has also been reported by Nelson.[2] In all of these experiments the androgen has been administered in regions of the body distant from the testes. It seemed not improbable that androgen of testicular origin (testosterone) might

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* Aided by a grant, administered by Dr. P. E. Smith, from the Rockefeller Foundation.

[1] Walsh, E. L., Cuyler, W. K., and McCullagh, D. R., *Am. J. Phys.*, 1934, **107**, 508.

[2] Nelson, W. O., *Anat. Rec.*, 1941, **79**, Suppl., 2, 48.

TABLE I.

Type of pellet		Days treated	Decrease in pellet wt (mg)	No. of animals	Vent. prostate wt, avg and range (mg)	Wt of treated testis, avg and range (mg)	Wt of untreated testis, avg and range (mg)
Cholesterol		20	0.00	3	23 (17-29)	289 (270-312)	296 (279-319)
Testosterone		20	4.46	4	276 (only 1 weighed)	885 (769-990)	868 (758-978)
"	33%	20	0.73	10	161 (81-269)	851 (642-1093)	570 (391-827)
"	33%	20	0.73	11	164 (65-250)	524 (338-715)*	507 (351-699)
"	15%	20	0.25	6	94 (54-110)	746 (674-948)	493 (420-617)
"	5%	20	0.05	6	57 (37-78)	556 (467-649)	408 (342-475)
"	20%	40	0.42	2	52 (51-52)	412 (392-431)	203 (202-203)
Untreated normal controls				45	186 (50-310)	1318 (795-1655)†	1302 (758-1638)
Untreated hypophysect. controls				5	16 (8-26)	228 (144-346)†	235 (136-355)

* The pellets were implanted in the subcutaneous tissue of the flanks, not in the testes.

† No pellets implanted.

be quantitatively more effective if administered intratesticularly because of a local or regional action. The greater efficacy of local application of hormones is well established in many conditions. Also, intratesticular administration would simulate, though crudely, the normal liberation of the hormone. It also might furnish evidence as to whether or not androgen in maintaining spermatogenesis acts directly on the testis. The extratesticular administration heretofore employed may be responsible for the relatively high dosages of androgen (usually 1 to 3 mg per day) required to maintain spermatogenesis in hypophysectomized rats.

In the experiments here reported, testosterone was administered intratesticularly by means of pellets.[†] The pellets were placed just beneath the tunica albuginea of the testis. A wide range of dosages, most of which were much lower than those heretofore reported, were used. In order to secure these low dosages of testosterone, its absorption was slowed by mixing it with cholesterol in varying proportions to produce mixtures containing 5, 10, 15, 20, 25, and 33% testosterone. Pellets containing only testosterone and only cholesterol were also used. The pellets were cylindrical and measured 1.7 mm in diameter by approximately 2 mm in length. They were weighed to the nearest 0.1 mg, and ranged from 4-5 mg. All implantations were made at the time of hypophysectomy into rats of the Long-Evans strain. They were autopsied after 20 or 40 days. The rats were 70 to 120 days old at the beginning of the experiment, most of them being between 90 and 120 days old.

With pellets containing only testosterone, the weight and structure of the testis not receiving the implant was as well maintained as was the one into which the pellet had been inserted. The accessory sexual glands were much heavier than normal. With a pellet of 33% testosterone, the testis into which it had been implanted was heavier than the opposite testis; its seminiferous tubules were better maintained, but sperm were also present in the other testis. The ventral prostate was maintained at about normal weight. The most pronounced differential effects were obtained with pellets containing the lowest percentages of testosterone used, namely, 5%. With these pellets, the seminiferous tubules of the testis into which an implant had been made contained sperm, whereas those of the other testis showed no spermatozoa whatsoever as in the untreated and cholesterol implanted controls. In some cases the spermatozoa were present throughout the treated testis; in other cases they were more numerous or present only in the neighborhood of the pellet. There was thus a local or regional effect. The size of the ventral prostate was much less than normal. Since the largest of these pellets contained only 250 μg of testosterone, there could be during a 20-day period an average absorption per day of only 12.5 μg, even if all the hormone were absorbed. The complete absorption of all the testosterone in the pellets seems improbable for the melting point of the center was different from that of the periphery of the removed pellets. Furthermore, the weight loss of these pellets averaged but 0.05 mg (an average of 2.5 μg per day), though the errors due to weighing and the absorption of substances from the body fluids makes an exact determination of the weight loss impossible.

[†] The testosterone was kindly supplied by Dr. Erwin Schwenk of the Schering Corporation.

The local or regional maintenance effected by the lower dosages indicates that testosterone acts directly on the testis and not by scrotal or general systemic stimulation.

Summary. Very small dosages of testosterone, administered intratesticularly to hypophysectomized rats, cause a regional maintenance of spermatogenesis. These dosages are much less than those necessary for complete maintenance of the sexual accessory glands. Testosterone has a direct action on the testis in the maintenance of spermatogenesis.

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Factors Influencing Ability to Counteract Gravitational Interference with Circulation, in the Rabbit.

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This is a report of a comparative study, in the rabbit, of a series of substances and procedures affecting ability to counteract the gravitational interferences with circulation imposed by the standing position. The substances studied were: paredrine, ephedrine, d-amphetamine, coramine, nicotinic acid amide, ascorbic acid, desiccated thyroid, sulfanilamide, sulfathiazole, and sodium nitrite. The procedures studied were: chilling, salt restriction, and bleeding from the heart and ear.

Method. New Zealand White male rabbits were used, between 3.0 and 4.5 kg in weight. The rabbits were chilled, in cool water,[1] until the rectal temperature had fallen to a point between 94 and 95°F. They were then dried and allowed to warm up again, spontaneously. When the rectal temperature had reached 96°F, they were placed in boxes forcing the rabbits to stand upright for one-half hour.

During this half hour of forced standing, temperature recovery proceeded without retardation in the rabbits able to counteract the gravitational interferences with circulation imposed by the standing position. In rabbits with an impaired capacity for counteracting gravitational interference, the rate of temperature recovery slowed down during the period of forced standing and, in extreme instances, reached negative values (impending gravitational shock).[1]

The extent of the temperature rise during the half hour of forced standing was measured: (1) in the absence of preliminary preparations other than were required to provide a true control for the test determination, and (2) one week later, during the period of action of the substance or procedure under study. Whenever practicable, an additional control determination was done one or 2 weeks after the test determination and averaged with the earlier control determination as a way of minimizing the magnitude of error due to spontaneous change.

An average test rise significantly greater than that observed during the control determinations was considered to be an indication of possible improvement as a result of the action of the substance or procedure tested. A significant decrease in rise was taken as an indication of converse impairment. A change

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[1] Locke, A., Locke, R. B., McIlroy, A. P., and Chung, L., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 184.

TABLE I.
Effect of Ingested Sodium Nitrite on Susceptibility to Gravitational Shock in the Rabbit. Partial Counteraction by Paredrine and by d-Amphetamine. Absence of Counteraction by Coramine or Nicotinic Acid Amide.

		Avg temp. rise in degrees F during the half hour of forced standing		
Substance given	No. of rabbits	Control detn.	Test detn.	Change
A, alone	24	1.84†	0.88*	—0.96 ± .11‡
A plus B	10	.48*	1.44	+ .96 ± .16
A " C	10	.64*	1.47	+ .83 ± .16
A " D	10	.54*	.51	— .03 ± .10
A " E	5	.14*	— .08	— .22 ± .18

* Nitrite alone given. † No nitrite given. ‡ The probable error of the difference, as computed from Peter's formula.

A: Sodium nitrite, 60 mg/kg by mouth as a 20% solution in water. The nitrite was given approximately 20 min before subjection to the half hr of forced standing.

B: Paredrine hydrobromide, Smith, Kline and French, 2.5 to 7 mg by vein immediately prior to the interval of forced standing.

C: d-Amphetamine sulfate, Smith, Kline and French, 2.5 to 7 mg as described under B.

D: Coramine, Ciba, 75 mg as described under B.

E: Nicotinic acid amide, Breon, 10 mg as described under B.

All of the rabbits were subjected to a preparatory chilling on the day preceding test.

in rise, regardless of magnitude or sign, was not considered to be significant unless it was substantially more than 3 times as large as the modifying "probable error."

Sodium nitrite. Ingestion of this substance in sufficiently massive amount is known to produce effects in man directly attributable to a lessened capacity for counteracting gravitational interference with the blood supply to the heart and brain.[2] A parallel effect on the rabbit is indicated in Table I. Sodium nitrite affected 3 of the 24 rabbits in Group A so severely that a fall in body temperature was registered during the half hour of enforced standing. A corresponding proportion was so little affected that temperature recovery proceeded at a normal rate, despite the gravitational handicap of the erect position. The majority were affected to the intermediate extent described by the average. The differences were, in part, individual and not due altogether to chance or to differences in weight. Only those rabbits clearly impaired by the nitrite were used for the further tests for capacity for offsetting action (Groups B, C, D, and E). The control averages for these latter groups are, for this reason, necessarily lower than the test average for Group A alone, and non-identical.

Paredrine. Experimentation by Kunkel, Stead and Weiss[2] led to a recommendation of paredrinol as an ideal agent for offsetting nitrite effect. Paredrine was found by Nathanson and Engelberg[3] to have a pressor action greater and more prolonged than that exerted by paredrinol. Paredrine produced a virtually complete counteraction of nitrite effect in the rabbit (Table I) and the parallel, partial counteraction of spontaneously heightened susceptibility to gravitational interference reported in Table IV.

Ephedrine. Ephedrine appeared to be equivalent to paredrine as an agent counteracting spontaneously heightened susceptibility to gravitational interference with circulation (Table IV).

d-Amphetamine. * The threshold dose of d-Amphetamine exerting central stimulation in the rat was found by Schulte and associates to be but one hundredth of that required by paredrine.[4] d-Amphetamine appeared to be as effective as paredrine vs. nitrite, in the rabbit (Table I), but ineffective against spontaneous-

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[2] Kunkel, P., Stead, E. A., and Weiss, S., *J. Clin. Invest.*, 1939, **18**, 679.

[3] Nathanson, M. H., and Engelberg, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 239.

* This substance was tested at the suggestion of Dr. A. C. Ivy.

[4] Schulte, J. W., Reif, E. C., Bacher, J. A., Lawrence, W. S., and Tainter, M. L., *J. Pharm. and Exp. Therap.*, 1941, **71**, 62.

TABLE II.
Effect of Blood Loss. Partial Counteraction by Ascorbic Acid.

Preparation	No. of rabbits	Avg temp. rise in degrees F during the half hour of forced standing		
		Control detn.	Test detn.	Change
F, alone	17	1.75*	0.55†	—1.20 ± .18
G, ''	26	1.99*	1.10†	— .89 ± .09
F plus H	7	— .24†	.93	+1.17 ± .25
G '' H	15	.81†	1.57	+ .76 ± .08

* No blood drawn. † Blood drawn, no ascorbic acid given.

F: 30 cc of blood drawn from the heart, 1 hr before the subjection to forced standing.

G: 30 cc of blood drawn from the marginal vein of the ear, 1 hr before the subjection to forced standing.

H: Ascorbic acid, Pfizer, 300 mg by vein immediately after solution in water and neutralization with sodium bicarbonate and immediately before the subjection to forced standing.

The control determinations for F, alone, and G, alone, were made 1 week before and after the test determinations. Those for F plus H and G plus H were made 2 weeks before and/or after. The rabbits were given a preparatory chilling on the day preceding test and weighed between 3 and 4.5 kg.

ly impaired capacity for counteracting gravitational interference with circulation (Table IV).

Coramine. Gunther, Engelberg and Strauss found that a lowered intramuscular pressure is not altered following paredrine but is consistently increased, together with the venous pressure, following coramine.[5] Coramine possibly was more effective than paredrine vs. spontaneous impairment in ability to counteract gravitational effect, in the rabbit (Table IV), but appeared to be ineffective vs. nitrite (Table I)—an antithesis of the picture found for d-Amphetamine.

Nicotinic acid amide. This vitamin with a chemical structure similar to that of coramine was given in considerably smaller amount. In the dosage given, it was ineffective vs. nitrite (Table I) and of only doubtful effectiveness vs. spontaneously induced impairment (Table IV).

Blood loss. The bleedings from the heart (Table II) had a greater impairing effect than the bleedings from the ear but were more hazardous for the rabbit. A wide range of effect was noted. Only the rabbits clearly impaired were used for the further tests for counteraction by ascorbic acid.

Ascorbic acid. Ascorbic acid has been reported to prolong the survival time following lethal injury[6] and blood loss[7] and to have effects on the water balance[8] possibly pertinent to the findings reported in Table II and IV. For the rabbits there described, ascorbic acid was definitely effective vs. spontaneous impairment in capacity for counteracting gravitational interference and markedly effective vs. impairment as a result of blood loss.

Sodium chloride. Excessive reduction in the salt intake and excessive loss of sodium chloride in the sweat are known to produce an impaired tolerance for the erect position in man.[9] Table III reports an equivalent, lessened capacity for counteracting gravitational interference with circulation in rabbits deprived of access to a salt lick, and a heightened capacity for such counteraction following restoration of access to salt.

Spontaneous impairment. None of the substances and procedures listed in Table IV appeared to produce more than a lessening of the pre-existing, spontaneous impairment present. None appeared able to confer on the rabbit a tolerance for the erect position in excess of that normally manifested in the absence of impairment. The more nearly the control rise approached a value of 2 or higher, the less frequently a heightened rise was ob-

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[5] Gunther, L., Engelberg, H., and Strauss, L., *Am. J. Med. Sc.*, 1942, **204**, 266, 271.

[6] Ungar, G., *Nature*, 1942, **149**, 637.

[7] Stewart, C. P., Learmouth, J. R., and Pollock, G. A., *Lancet*, 1941, **240**, 818.

[8] Reid, M. R., *Physiol. Rev.*, 1943, **23**, 76.

[9] Nadal, J. W., Pedersen, S., and Maddock, W. G., *J. Clin. Invest.*, 1941, **20**, 691.

TABLE III.
Effect of Change in Salt Intake.

Preparation	No. of rabbits	Avg temp. rise in degrees F during the half hour of forced standing		
		Control detn.	Test detn.	Change
Salt lick put in cage	12	1.53	2.08*	+0.55 ± .13
taken out	16	2.11	1.38†	— .73 ± .11
returned	14	1.01	1.66†	+ .65 ± .07

* 2 to 7 days after the placement of the salt rings in the cages and 6 days or longer after the last preceding determination.

† 6 to 14 days after the change in salt intake indicated.

The rabbits were given a preparatory chilling on the day before test and were given no salt other than that in the salt rings, excepting for the amounts naturally present in their food (hay and oats plus small amounts of bread and carrot).

TABLE IV.
Direct Effects of Paredrine, d-Amphetamine, Ephedrine, Coramine, Nicotinic Acid Amide, Ascorbic Acid, Desiccated Thyroid, and Chilling.

Substance or preparation given	Amt, mg	No. of tests	Avg temp. rise in degrees F during the half hour of forced standing		
			Control detn.	Test detn.	Change
Rabbits with a control rise of 0 to 1.3 degrees:					
None		18	0.88	1.05	+0.17 ± .07
B	2.5 to 15	14	1.00	1.41	+ .41 ± .10
C	.5 to 10	11	.74	.72	— .02 ± .13
J	2.5 to 5	13	.96	1.40	+ .44 ± .11
D	37 to 75	18	.84	1.53	+ .69 ± .13
E,E′	10 to 20	27	.87	1.11	+ .24 ± .09
H′	300	11	.66	1.27	+ .61 ± .12
L		16	.93	1.54	+ .61 ± .09
M′		13	.89	1.92	+1.03 ± .10
M″		20	.84	1.64	+ .80 ± .11
Rabbits with a control rise of more than 1.3 degrees:					
None		11	1.85	1.88	+ .03 ± .07
B	2.5 to 15	20	2.04	1.93	— .11 ± .13
C	.5 to 5	20	1.84	1.46	— .38 ± .10
D	75	14	1.71	2.06	+ .35 ± .12
E,E′	10 to 20	16	1.81	1.92	+ .11 ± .07
H′	300	18	1.86	2.02	+ .16 ± .08
L		9	1.81	1.94	+ .13 ± .04
M′		8	2.06	2.25	+ .19 ± .07
M″		22	1.92	2.31	+ .39 ± .11

B, C, D, E: See Table I.

E′: Nicotinic Acid, Eastman, as described under B.

H′: Ascorbic Acid, Pfizer, by mouth, 1½ hours before the subjection to forced standing.

J: Ephedrine sulfate, Mallinckrodt, as described under B.

L: Subjection to preparatory chilling on the preceding day. No chilling had been given for one week or longer preceding the control determinations in this group. A chilling on the preceding day, following one week without chilling, was given for all of the determinations in H′, since ascorbic acid has appeared to be ineffective in the absence of such preparation.[1] Whether or not a preceding chilling had been given appeared to be less material for the other agents in this table. Each test determination was, excepting for group L, done exactly as the controls (that is, if a preparatory chilling was given on the day before the control determination, an equivalent chilling was given, also, on the day before the test determination).

M′: Desiccated Thyroid, Parke-Davis, 200 to 300 mg by mouth daily for 8 days. The thyroid feedings at this level produced an average weight loss of 0.3 kg and death within 5 to 8 days in the proportion of 5/18.

M″: Desiccated Thyroid, 100 mg by mouth daily for 7 to 13 days. Supplementary feedings of ascorbic acid and of vitamin B complex were given to a few of the rabbits in this group, without observed material effect on outcome. The feedings at this level were better tolerated, producing no deaths and an average total weight loss of only 0.09 kg.

TABLE V.
Effects of Sulfanilamide and Sulfathiazole.

Substance given	Amt, g/kg	Avg blood level, total mg %	No. of rabbits	Avg temperature rise, F, during the half hour of forced standing: Control detn.	Test detn.	Change
SA	0.11 to .19	7.7	12	1.48	1.14	—0.34 ± .07
	.20 to .29	14.1	7	1.99	1.44	— .55 ± .24
ST	.11 to .23	6.0	7	2.20	2.21	+ .01 ± .08
	.32 to .48	8.8	5	1.96	1.66	— .30 ± .12

SA: Sulfanilamide, Winthrop, by mouth 3 hours before the subjection to forced standing.
ST: Sulfathiazole, Maltbie, by mouth as described for SA.

served after administration of the substance or procedure under test.

Hardening. Hardening has been suggested as a corrective measure vs. the impaired capacity for counteracting gravitational interference associated with effort syndrome.[10] Both exercise and chill appear to contribute to the hardening process. One preliminary chilling to 94°F on the day preceding test produces a lessening of impairment so large, in rabbits unsubjected to chilling for a week or longer preceding test,[1] as to require the rigorous control for this factor maintained throughout the tests made for this report.

Desiccated thyroid. The effectiveness of this principle vs. spontaneous impairment in ability to counteract gravitational interference with circulation, in the rabbit, already has been indicated in reference 1.

Sulfanilamide, sulfathiazole. The rabbits tested for effect by these drugs were not harboring infection to an extent producing observed effect on weight or temperature—a qualification of importance because infection lessens the tolerance for the upright position[1] and sulfa drugs are not ordinarily given in the absence of infection. Virtually no impairment was apparent from the amounts of sulfathiazole given, during the time covered by the tests. Moderate impairment was observed with the sulfanilamide.

Summary. The following substances and procedures have been observed to have effects on the rate of temperature rise in chilled rabbits confined to an erect position (1) indicative of tendency to produce impairment in ability to counteract gravitational interference with circulation: blood loss, decreased salt intake, "softening" inaction, infection, sodium nitrite, and, to a considerably less marked extent, sulfanilamide; and (2) indicative of tendency to produce a converse lessening of pre-existing impairment: paredrine, ephedrine, coramine, ascorbic acid, thyroid, and hardening. Paredrine had a parallel effect vs. nitrite-induced impairment. Coramine, effective vs. spontaneous impairment, appeared to be ineffective vs. nitrite-induced impairment. d-Amphetamine, in direct contrast, gave indications of effectiveness vs. nitrite-induced impairment but not vs. spontaneous impairment. Impairment as a result of blood loss was substantially lessened following intravenous sodium ascorbate, in massive dosage.

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[10] Lewis, T., *The Soldier's Heart and the Effort Syndrome*, 1919.

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Effect of Protein Extracts of Neural Plate Plus Chordamesoderm on Presumptive Epidermis.

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The purpose of these investigations was to test crude protein extracts of the neural plate plus chordamesoderm for ability to induce neural tubes in the presumptive epidermis of three species of Amblystoma. The work was undertaken because the neural inductions obtained by protein residues after ether extraction, as described by Holtfreter,[1] were larger and better organized than those obtained with the ether soluble fractions as observed by Needham, Waddington, and Needham[2] or Barth.[3]

Extraction methods. One ml of neural plates plus chordamesoderm was extracted with 11 ml of 5% NaCl for 24 hours at 5°C. Stage 15 (Harrison stages) of *Amblystoma punctatum* was used for donors. The extract was then filtered through a Seitz filter and treated in 2 ways before using. Some was diluted with Holtfreter's solution which caused a precipitate to form. This precipitate with the solution was injected into the blastocoel of stage 10 (Harrison stages) by means of a micropipette. Another portion of the extract was dialized at 5°C and the resultant precipitate was injected.

The residue after extraction with NaCl was then further extracted with 0.05 N NaOH at 5°C and passed through a Seitz filter. Acetic acid was added to precipitate the proteins and this precipitate was injected using a micropipette or implanted by means of a forceps into the blastocoel.

The chloroform extract was made by shaking ground-up neural plate plus chordamesoderm with chloroform until a chloroform-protein gel was obtained. This gel was separated by centrifuging and the chloroform removed at 5°C. The residue was implanted into the blastocoel by means of forceps.

Buffer extracts were made with phosphate citric acid buffers at pH 7 by freezing and thawing tissues in centrifuge tubes. After centrifuging the clear layer of buffer solution was removed and injected.

Material. The presumptive epidermis of the gastrulæ stage 10 and 11 of *Amblystoma punctatum*, *A. opacum*, and *A. mexicanum* was used to test the inducing ability of the extracts. The proteins were usually precipitated from the extracts and the precipitate was then drawn up into a glass pipette controlled by means of a micromanipulator. The pipette was inserted through the vitelline membrane and into the blastocoel and a measured amount injected. The reactions of the presumptive epidermis were classified as (1) induction of a neural tube and (2) induction of aggregations of neural cells. Fig. 1, A, B, C, illustrates the variation in the neural tubes which were induced by extracts.

Results. Table I compares the effects of 5% NaCl extracts, 0.05 N NaOH extract, chloroform extract, and neutral buffer extract of the neural plate plus chordamesoderm. It is clear that only the NaOH extract induces neural tubes in appreciable numbers. NaCl extracts induced only one clear neural tube and 3 neural aggregations. The chloroform extract induced no neural tubes but consistently induced large masses of epidermal cells to aggregate around the implant forming a large growth on the side of the embryo. Buffer extract gave no consistent reaction except that it tended to produce cytolysis in a number of cases.

Comparison of extracts of anterior with posterior neural plate plus chordamesoderm. For this comparison ten embryos in early neural

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[1] Holtfreter, J., *Arch. Entw.-mech.*, 1934, **132**, 226.

[2] Needham, J., Waddington, C. H., and Needham, D. M., *Proc. Roy. Soc. London B*, 1934, **114**, 393.

[3] Barth, L. G., *Biol. Bull.*, 1934, **67**, 244.

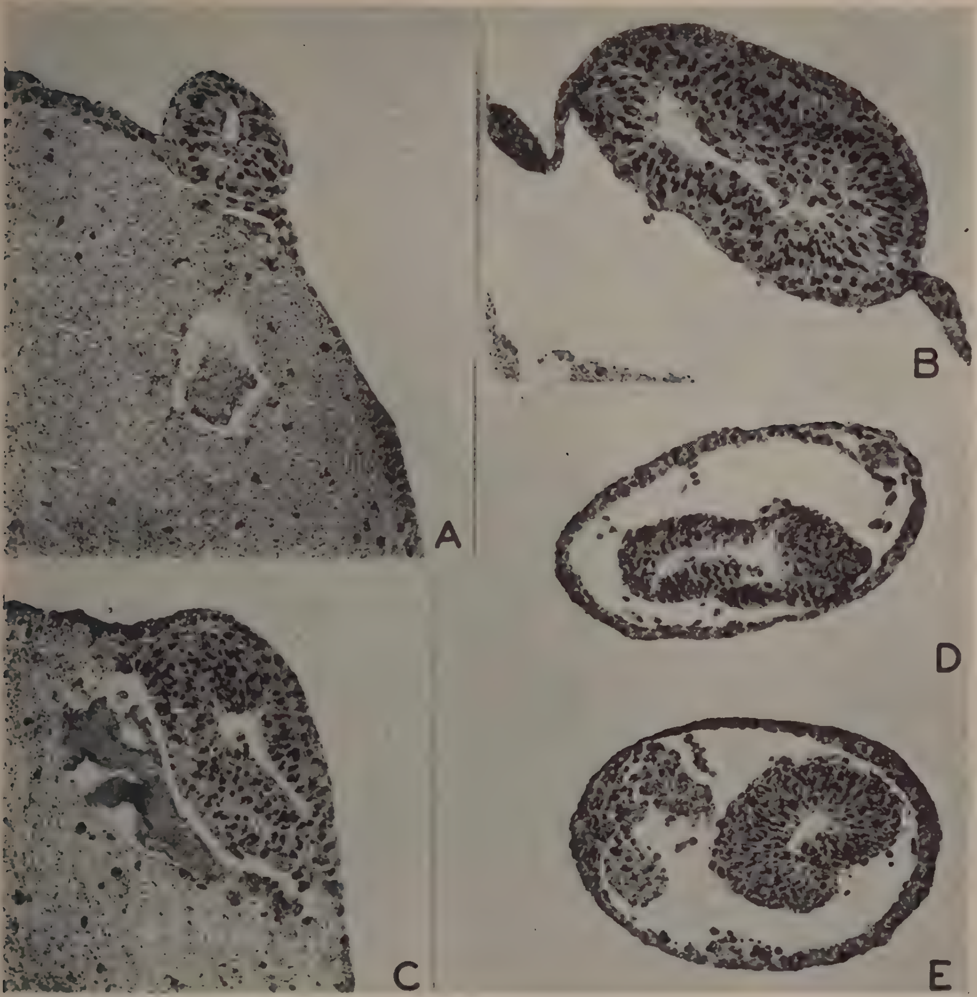

FIG. 1.
A, small neural tube induced in the ventral epidermis of the whole embryo by protein extract; B, C, large neural structures induced by protein extracts. D, E, neural structures induced in explants of the presumptive epidermis by frozen-dried preparations of the organizer.

TABLE I.
Comparison of Crude Protein Extracts.

Extract	No. cases	Neural tube	Neural aggregat
5% NaCl (diluted)	69	1	0
5% ” (dialized)	35	0	3
0.05 N NaOH	69	26	9
Chloroform	48	0	1
Buffer pH 7	26	0	0

plate stage were prepared by removing the membranes and cutting out the neural plate together with the adhering roof of archenteron. These preparations were then divided into equal thirds by transverse cuts, allowing for the difference in width. The anterior and posterior thirds were used for extraction while the middle third was discarded.

The anterior and posterior thirds were then treated in exactly the same way. The water was pipetted off and 65 mm^3 of .05 N NaOH added and immediately frozen in a mixture of ether and solid CO_2. While still frozen the tissue was ground up by means of a steel drill. The preparation was allowed to thaw and the process of freezing and grinding repeated. After thawing the preparation was centrifuged for 2 minutes at 10,000 R.P.M. This gave a layer of fat on the top and a layer of yolk granules and pigment on the bottom of the tube with a clear solution between. The clear fluid was pipetted off and the proteins precipitated with 65 mm^3 of .05 N acetic acid. If the drop of acetic acid is added gently without stirring, a firm precipitate is obtained which can be cut into fragments, picked up with a forceps and inserted into the blastocoel. After the precipitate formed, a few drops of Holtfreter's solution was added and the resultant pH was measured by the Beckman microglass electrode assembly. The pH was varied by addition of .05 N acid or alkali. The amount of precipitate which was implanted varied but averaged about 2 μg dry weight per embryo.

TABLE II.
Comparison of Extracts of the Anterior with Posterior Neural Plate Plus Chordamesoderm.

	pH	No. cases	Neural tubes	Neural aggregates
Anterior	6.5-7.0	30	7	4
Posterior	5.5-6.5	29	6	1
Anterior	7.5-8.0	40	17	1
Posterior	7.5-8.0	29	19	1

Results are given in Table II. Experiments in which the pH after precipitation with acetic acid was brought to 7.5 or 8.0 gave the best results. Observation of the living neurulæ showed small neural plates which rolled into tubes in the normal fashion. In some cases the injection mass broke up into 3 fragments and 3 small plates could be seen to fold into small tubes. The sections were studied carefully to see if there were any differences in the kind of neural tube induced by anterior as compared with posterior extracts and no differences could be detected. Both anterior and posterior extracts induced small neural tubes as in Fig. 1, A, and also large brain-like tubes as in 1, B. 1, C, shows clearly the protein precipitate adjacent to the induced neural tube. All of these tubes are completely independent of the normal primary neural tube which is not shown in the figure.

The effect of frozen dried preparations of the neural plate plus chordamesoderm on presumptive epidermis. In order to test the proteins in as nearly a native state as possible frozen dried preparations of the neural plate plus chordamesoderm were made by freezing neurulæ stage 15 (Harrison stages) in tubes immersed in ether-solid CO_2 mixtures followed by desiccation at low pressures. These dead dried neurulæ were very well preserved showing little or no shrinkage and the neural plate plus chordamesoderm was dissected out and applied to the inner (blastocoel) surface of explants of the normal presumptive epidermis of stage 10-11. The edges of the explant curl forming a cup-shaped preparation with the dried tissue on the inside.

Table III records the results of a comparison of the action of anterior and posterior dried preparations of the neural plate plus chordamesoderm and dried epidermis. Explants of the living presumptive epidermis having had no treatment with dried tissue were used as controls. Both anterior and posterior dried preparations induce neural tubes Fig. 1, D, E, and a careful study of the sections revealed no differences in the structure of the induced neural tubes. Dried epidermis also induced neural tubes which did not differ from the above. Controls with no treatment formed some neural tubes and this has been reported in detail by Barth.[4] Quantitatively the posterior neural plate plus chordamesoderm induced the highest percentage of neural tubes (31%), the epidermis was next with 26%, the anterior neural plate plus chordamesoderm gave 18% induction while the non-treated controls formed neural tubes in 13% of the cases.

TABLE III.
Reaction of Explants of the Presumptive Epidermis to Frozen Dried Tissues of the Neurula.

Tissue	No. cases	Neural tube	Neural aggregate
Anterior	37	7	2
Posterior	32	10	3
Epidermis	15	4	4
No tissue	38	5	1

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[4] Barth, L. G., *J. Exp. Zool.*, 1941, **87**, 371.

Summary. Protein extracts of the neural plate plus underlying chordamesoderm induce the presumptive epidermis of the gastrula to form neural tubes. However no differences were obtained in the structure of the neural tubes which were induced by extracts of anterior as compared with posterior regions of the neural plate and underlying chordamesoderm. Preparations of the anterior and posterior regions of the neural plate and underlying chordamesoderm which were killed by freezing and subsequent drying induced neural tubes in the explanted presumptive epidermis of the gastrula. Quantitative differences in the frequency of induction were obtained with the posterior neural plate and underlying chordamesoderm inducing with the greatest frequency.

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Further Report on Malignancy Observed in Rats Injected with Crude Ether-Extracted Wheat Germ Oil.

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In previous papers from this laboratory[1-3] the presence of a carcinogenic substance has been demonstrated in crude ether-extracted wheat germ oil. An interesting observation is that some of these tumors developed from feeding this oil, either by a forced feeding method or by mixing the oil with the diet. However, some were also produced by intraperitoneal injection of the oil. Since that time, several investigators[4-9] have attempted to produce neoplasms by the feeding of similar oils, but all have been unsuccessful. However, nothing has been further reported upon the production of malignant tumors by injection of a crude wheat germ oil.

In the past 3 years our attempts to produce tumors by feeding crude ether-extracted wheat germ oil have, as with other investigators, resulted in failure. On the other hand, by injection we have been able to produce a substantial number of tumors with some of the oils available, a fact which has been confirmed by the similar results of another investigator.[10]

Materials and Methods. Four strains of rats, Wistar, Buffalo, a hooded variety of our own breeding, and a strain of Wistar rat supplied by Eli Lilly and Company were used. Four different oils were used also. One was a percolated oil with the sediment removed, supplied by Eli Lilly and Company. This oil is identified by the letter L in the table. The 3 other oils were prepared by us by a Soxhlet type of extraction, one for 8 hours (8E), one for 12 hours (12E), and one for 24 hours (24 Ma). These were used as prepared, the sediment shaken each time before use, except that in one sample of the 24-hour oil the sediment was removed before use (24 MaC). Two series of control oils were also used. One was a purified expressed wheat germ oil supplied by Eli Lilly and Company. The other was sesame oil, divided into 3 parts, one part of which was used plain, another contained 2% of starch in suspension, and a third con-

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[1] Rowntree, L. G., Lansbury, J., and Steinberg, A., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 424.

[2] Rowntree, L. G., Steinberg, A., and Brown, W. R., *Tr. A. Am. Physicians*, 1937, **53**, 199.

[3] Rowntree, L. G., Steinberg, A., Dorrance, G. M., and Ciccone, E. F., *Am. J. Cancer*, 1937, **31**, 359.

[4] Auchinloss, R., and Haagensen, C. D., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 703.

[5] Brues, A., Marble, B., and Riegle, B., *Cancer Research*, 1941, **1**, 815.

[6] Carruthers, C., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 107.

[7] Ginzton, L. L., and Connor, C. L., *Am. J. Cancer*, 1940, **38**, 90.

[8] Halter, C. R., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 257.

[9] Rider, D. L., *Am. J. Cancer*, 1940, **38**, 275.

[10] Clowes, G. H. A., personal communication.

TABLE I.
Results of Injections of Crude Wheat Germ Oil.

No. of animals	Strain	Oil No.	No. of tumors	Type of tumor	Days elapsed before autopsy	% bearing tumors
1	Hooded	24 Ma	0			
2	Buffalo	24 Ma	0			
2	”	24 MaC	0			
2	Hooded	8E	0			
3	Wistar	8E	1	Ca	223	33
8	”	24 Ma	2	1 Ca †	545 674	25
3	”	24 MaC	0			
3	”	12E	0			
3	”	L	2	2 Sa	249, 353	66
10	” *	L	5	5 Sa	220, 289, 299 478, 588	50
2	” *	24 Ma	0			
2	” *	12E	1	Ca	671	50
3	”	Sesame	0			
3	”	Sesame, 2% starch	0			
3	”	Sesame, 2% starch, 2% sterols	1	Sa	555	33
5	”	Pressed wheat germ oil	0			
				Total rats		27
				Wistar rats only		32

* Of Purdue stock supplied by Eli Lilly & Company.
† Atypical cell tumor not definitely classified.

tained 2% of starch and 2% of wheat germ sterols. The purpose of these was to see if some of the known constituents of the wheat germ oil used had any effect.

Each animal received 1 cc of an oil intraperitoneally once a week until a palpable mass was detected in the majority of the animals. This required 4 injections. The oil was then given but once in 2 weeks. After about 30 injections the oil was usually exhausted, and the animals were allowed to live some time without further treatment. The usual laboratory diet was given throughout the experiment.

Results. After the first 4 injections of wheat germ oil there was palpable in the abdomen of all animals a large, rather firm mass. To determine what this mass was, 3 animals not included in this report were injected with crude wheat germ oil for 4 weeks and sacrificed on the fifth week. Autopsy revealed that the mass was caused by a peritonitis. The intestines, spleen, liver, and bladder were all enveloped and connected with fibrous adhesions giving a solid mass which could not easily be separated by blunt dissection. No considerable amount of oil was to be found anywhere within the mass.

The injections were carefully continued on the other animals. After 7 or 8 injections the mass as a rule became quite hard. After about 15 injections one-third of the animals developed a sinus penetrating through the peritoneal wall, and skin, which in some cases evidenced a purulent discharge and in some cases remained clean. No oil, however, could be found oozing from such wounds. These healed as a rule, leaving a hernia in a few cases. The injections were continued every other week unless the life of the animal seemed endangered when alternate injections were omitted. Malignancy was shown in animals that came to autopsy in from 220 to 670 days.

The control oils gave negative findings showing neither the peritonitis nor the lesions which were obtained with the crude wheat germ oil.

Table I gives in detail the final results of this series of experiments.

Malignant tumors were obtained in the two Wistar strains of rats only. Combining these, a total of 32% of the Wistar animals on all crude wheat germ oils developed malignant tumors. It is worthy of note perhaps, that the one sesame oil control rat which also succumbed to a malignant tumor, was one in which 2% of wheat germ oil sterols were dissolved and 2% starch was suspended.

The control animals lived as long as the experimental animals (353 to 647 days). There were no spontaneous tumors throughout the colony of over 500 rats from which most of the experimental animals were taken.

One experimental animal was sacrificed after 362 days and the tumor found carried through 6 generations of transplants.

Discussion. It is believed that these results definitely indicate the presence of a carcinogenic substance in crude ether-extracted wheat germ oil. Because of the presence of adhesions within the abdomen it might be argued that the malignancy was caused solely by irritation. But, if one considers that while all of the crude wheat germ oils used produced similar adhesions, yet the percentage of actual malignancy varied widely with different oils, this conclusion appears unwarranted. Indeed, this variation of tumor growth with different batches of crude wheat germ oil made by extraction with ether, lends support to our belief that a carçinogenic substance is present in some oils and absent in others. We are, however, unable to state as yet all the conditions essential to reproduce consistently these results with a wheat germ of any source.

Summary. Malignant tumors have been produced in, on an average of, 32% of Wistar rats injected with crude wheat germ oil made by extraction with ether. With one batch of oil, tumors resulted in 66% of the injected rats. The tumors were preponderantly sarcomas but carcinomata were induced in 3 rats.

We are indebted to Dr. Eugene A. Case of the Graduate Hospital of the University of Pennsylvania for the preparation of the pathological specimens and reports on the microscopic studies of the tumors, and to Miss Madeline Hemphill for technical assistance in carrying on the experiments. We wish to express grateful acknowledgment to the American Philosophical Society fôr financial support of the work from the Judson Daland Fund.

14330

Effect of Pseudopyridoxine on Rat and Yeast Growth.*

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In a recent report Snell, Guirard, and Williams[1] demonstrated the presence of a substance in natural products which surpasses pyridoxine in its physiological activity on *Streptococcus lactis* cultured on a pyridoxine-free medium. The substance had properties similar to pyridoxine, hence was called pseudopyridoxine. Tissues and urine were found to contain pseudopyridoxine, the amount depending on the quantity of pyridoxine ingested. These workers suggested that the compound is a metabolite of pyridoxine. Snell[2] showed that pseudopyridoxine was formed when pyridoxine was autoclaved with the basal culture medium for *S. lactis*. The amount formed increased as the period of heating was increased, and was due to the interaction of pyridoxine with amino acids during autoclaving.

Before the paper of Snell *et al.*[1] appeared it had been observed in this laboratory that the recovery of pyridoxine after various chemical treatments was very high as measured by the *Lactobacillus casei* method of Landy and Dicken.[3] Since this test organism responds to pseudopyridoxine in a manner

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[1] Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

[2] Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 356.

TABLE I.

Pyridoxine and Pseudopyridoxine Content of Preparations.

Preparation	*L. casei* assay value (pyridoxine plus pseudopyridoxine)	Yeast assay value (pyridoxine)	Pseudopyridoxine content
	Micrograms per cc or g		
Acid autoclaved yeast extract	1617	17	1600
Pyridoxine hydrochloride, cystine treated, equivalent to 5 γ B_6 per cc	9.4	4.6	4.8
Pyridoxine hydrochloride, peroxide treated, equivalent to 5 γ B_6 per cc	24	4.6	19.4

similar to *S. lactis*[2,4] the active substance formed on chemical treatment was probably pseudopyridoxine.

It is apparent that pseudopyridoxine has some physiological significance in the metabolism of microörganisms. It seemed important to decide as definitely as possible whether it would also promote growth of higher animals, and of the strain of yeast used for pyridoxine assay.[5] Experiments designed to provide this information were therefore undertaken.

Experimental. Pseudopyridoxine determinations were made by a differential assay procedure to be described in detail elsewhere.[4] This method involves a preliminary estimation of both pyridoxine and pseudopyridoxine with *L. casei* followed by determination of pyridoxine alone by the yeast method.[5] For convenience, pseudopyridoxine is expressed in the terms used by Snell *et al.*, namely, microgram equivalents of pyridoxine.

Solutions containing pseudopyridoxine were prepared by the following 3 methods: (a) Cystine treatment. A solution of 250 μg of pyridoxine hydrochloride, 50 mg cystine hydrochloride, and 250 mg sodium acetate in 50 cc of water at pH 7.2 was autoclaved for 1 hour at 15 lb pressure. (b) Peroxide treatment. Two hundred fifty micrograms of pyridoxine hydrochloride in 40 cc of water were treated with 10 cc of 3% hydrogen peroxide for 4 hours at room temperature. The excess peroxide was removed with manganese dioxide, the solution filtered, and the filter paper washed thoroughly. (c) Heat treatment. Fifteen grams of Difco yeast extract dissolved in 50 cc of 0.1 N hydrochloric acid were autoclaved at 15 lb pressure for 1 hour. The solution was neutralized for assay.

The pyridoxine and pseudopyridoxine content of the 3 preparations are listed in Table I. Although some uncertainty attaches to the high pseudopyridoxine value listed for autoclaved yeast extract,[4] it seems very probable that a large fraction of this value actually represents pseudopyridoxine. Since the last 2 preparations were made from synthetic pyridoxine and 92% of the pyridoxine was recovered as determined by the yeast assay method, it is apparent that the pseudopyridoxine also present in the solutions did not stimulate yeast growth, and that most of the pyridoxine was unchanged by these treatments.

The effect of pseudopyridoxine on the growth of rats was determined by assaying the above preparations by the method of Conger and Elvehjem.[6] The pyridoxine content of the unheated yeast extract was found by the rat assay to be 15 μg per gram (Table II) while that of the acid autoclaved material was essentially the same. Both of these results are in good agreement with the yeast assay value (Table I). Acid autoclaved yeast extract, therefore, did not cause enhanced growth in the rat.

That pseudopyridoxine does not stimulate rat growth is more clearly demonstrated by

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[3] Landy, M., and Dicken, D. M., *J. Lab. Clin. Med.*, 1942, **27**, 1086.

[4] Carpenter, L. E., and Strong, F. M., *Arch. Biochem.*, in press.

[5] Atkin, L., Schults, A. S., Williams, W. L., and Frey, C. N., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

[6] Conger, T. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, **138**, 555.

TABLE II.
Effect of Pseudopyridoxine on Rat Growth.

No. rats	Supplement to basal	Amt per day	Avg gain per wk, g	Pyridoxine hydrochloride content, γ/ cc or g
3	None		7.8	
3	Pyridoxine hydrochloride	5 γ	24.0	
3	Difco yeast extract	0.33 g	24.0	15
3	Difco yeast extr. acid autoclaved	≎ 0.33 g	26.0	*ca.* 16
3	Pyridoxine hydrochloride, cystine treated	≎ 5 γ	20.0	3.8
3	Pyridoxine hydrochloride, peroxide treated	≎ 5 γ	22.0	4.2

TABLE III.
Effect of Pseudopyridoxine on Rat Growth.

No. rats	Supplement to basal	Amt per day	Avg gain per wk g	Pyridoxine hydrochloride content, γ/cc or g
5	None		6.8	
5	Pyridoxine hydrochloride	5 γ	23	
5	Difco yeast extract	0.33 g	27	*ca.* 18
5	Difco yeast extr., acid autoclaved	≎ 0.33 g	26	*ca.* 18
5	Pyridoxine hydrochloride, cystine treated	≎ 5 γ	19	3.8
5	Pyridoxine hydrochloride, peroxide treated	≎ 5 γ	20	4.1

the growth response of rats fed the cystine and peroxide treated pyridoxine preparations. Both preparations contained pseudopyridoxine, but the rats failed to show stimulated growth (Table II). The rat assay values for these preparations showed that 76 and 84% of the pyridoxine in the cystine and peroxide treated solutions respectively was unchanged by the chemical treatment.

The rat experiment was repeated using a larger number of animals per group and employing new pseudopyridoxine preparations. The hydrogen peroxide and cystine treated pseudopyridoxine preparations this time contained 22.0 and 12.8 μg equivalents of pseudopyridoxine and 4.7 and 4.9 μg of pyridoxine respectively. The results listed in Table III corroborate the previous findings.

Discussion. The above results indicate that pseudopyridoxine as found in natural materials or as prepared from pyridoxine by chemical agents does not stimulate yeast or rat growth. Since attempts to prepare solutions of pseudopyridoxine free from pyridoxine were unsuccessful,[4] it was impossible to determine whether or not pseudopyridoxine had any pyridoxine activity at all for these organisms.

Recently Robbins and Ma[7] have demonstrated that pseudopyridoxine does not affect the growth of certain molds that require pyridoxine for normal development. The fact that the determination of pyridoxine in natural materials by *Neurospora sitophila* gives results of the same order of magnitude as found by yeast and rat assay[4] suggests that this organism also does not respond to the pseudo form of the vitamin. From present evidence, therefore, it seems that the physiological activity of pseudopyridoxine is limited to the lactic acid bacteria.

Summary. Synthetic pyridoxine was converted to pseudopyridoxine by treatment with hydrogen peroxide and by autoclaving in a cystine solution. Pseudopyridoxine formed in this manner and that found in autoclaved yeast extract did not stimulate yeast or rat growth.

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[7] Robbins, W. J., and Ma, R., *Proc. Nat. Acad. Sci., U. S.*, 1943, **29**, 172.

14331 P

Anoxia Cataract.

John G. Bellows and Dorothy Nelson. (Introduced by C. J. Farmer.)

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The most vital requirement of tissue is an adequate supply of oxygen. Tissues possessing a blood supply are bathed in a medium containing 13 to 19 vol. % oxygen. This large amount is made possible by the hemoglobin of the red cells which holds the oxygen in a loose chemical combination. The amount of oxygen in physical solution in the body fluids is extremely small. For example, the aqueous humor has only from 0.08 to 0.12 vol. % oxygen (Friedenwald and Pierce[1]). The necessary rapid replenishment of aqueous oxygen is made possible by rapid diffusion from the ciliary body (Pierce, Friedenwald and Freeman,[2] and Friedenwald and Pierce[1]), and by the passage of oxygen from the atmosphere through the cornea (Fischer[3]). From these considerations it is not surprising that there should be adverse effects upon the lens because of a lack of oxygen, an assumption substantiated by our experiments.

Biozzi[4] reported the effects of asphyxia on rats placed singly into 3-liter bell jars. He noted the development of cataract in some of these animals and subsequent regression after a short time in the fresh air.

Experimental. We undertook investigations to determine whether anoxia alone would produce cataract. Rats were placed in a steel chamber in which the pressure was gradually reduced until it was equal to that at an altitude of 30,000 feet or more. When the conditions were severe enough so that 50% of the animals died, about 75% of the dead animals and about 10% of the surviving animals had lens opacities. That the cataracts were due to anoxia and not to pressure was demonstrated by placing rats singly in glass chambers arranged so as to permit the constant flow of a mixture of gases consisting of 5% oxygen and 95% nitrogen at sea level. Again, the rats showed lens opacities. These experiments indicate that cataracts can develop in anoxia and that asphyxia and pressure are not important factors.

Since it is known that the oxidation of the lens is mainly anaerobic and that the lactic acid content in the aqueous humor is ordinarily higher than in the blood, it was thought that lactic acid determination of the aqueous would be of interest. For this investigation rabbits, known to be very resistant to changes in altitude, were employed. The aqueous humor was removed from one eye of each animal under ether. After 2 hours allowed for recovery some were kept in cages as controls; the others placed in the decompression chamber. After 2 hours the latter group were brought down to normal level, anesthetized again, and the aqueous was removed from the second eye. Results were impressive. Whereas the average lactic acid content of the aqueous humor in all of the first eyes and in the second eyes of the controls ranged from 70 to 75 mg per 100 cc of aqueous (a value somewhat higher than normal, probably due to the effects of the ether anesthesia) the average lactic acid value of the aqueous in the second eyes of the decompressed animals was 3 to 4 times as high (250 mg per 100 cc).

In order to determine whether the lenticular changes are due to a shift in the pH of the aqueous, rabbits were decompressed to 30,000 feet. The aqueous humor was removed under oil and the pH determined by glass-electrode method. Since the pH of the aqueous was found to be unaltered, the opacities are due either to a specific action of the lactic acid or, more likely, to an osmotic upset. The latter was suggested as a possible cause by the work of Bellows and Chinn.[5]

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[1] Friedenwald, J. S., and Pierce, H. F., *Arch. Ophthal.*, 1937, **17**, 477.

[2] Pierce, H. F., Friedenwald, J. S., and Freeman, D., *Am. J. Physiol.*, 1933, **104**, 553.

[3] Fischer, F. P., *Arch. f. Augenheilkunde*, 1929, **102**, 146.

[4] Biozzi, G., *Arch. f. Ophthal.*, 1935, **133**, 423.

Ocular Findings. After decompression, there is a marked hyperemia of the iris. The light beam in the aqueous, although difficult to observe, is visible. With oblique illumination small diffuse gray opacities are observed in the superficial cortex. The most striking finding is the fact that the anterior superficial suture becomes more readily visible. By use of the higher magnification of the slit-lamp the changes are seen to begin with development of opacity of the anterior sutures from which fine fibrillar opacities extend. In optical section it is seen that the entire opacity is thin and lies in the superficial layers of the cortex. Gradually the opacity extends towards periphery. Similar changes, although less marked, occur in the posterior cortex. In severe cases the fiber structure is lost and a shiny white total cataract is formed.

The cataract lasts from about ¾ to 1 hour and then gradually regresses, exactly reversing the course of its formation, so that the last opacities to disappear are those about the sutures.

Although the author has examined the lenses of human volunteers subjected to decompression to the equivalent of 18,000 feet, it must be pointed out that opacities due to anoxia have been observed only in animals.

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[5] Bellows, J. G., and Chinn, H., *Arch. Ophthal.*, 1941, **24**, 979; 1941, **25**, 796.

14332

Distribution and Excretion of Atabrine in Experimental Animals.

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Although a number of workers have reported upon the absorption, distribution, and excretion of atabrine in experimental animals, current interest in this antimalarial drug suggested an extension of these studies.

Methods. The method used for the extraction of atabrine is a modification of Hecht's procedure[1]: One gram samples of tissue or feces were warmed in 10 cc of 30% sodium hydroxide on a water bath for 5 to 15 minutes to emulsify solids. The mixture was cooled and extracted 3 times with 5 cc portions of isoamyl alcohol, centrifuging after each extraction. Ten cc samples of urine were rendered strongly alkaline and extracted directly with isoamyl alcohol without prior heating. Oxalated blood samples were precipitated with 9 volumes of acetone and the acetone filtrates were evaporated to dryness and the residues were taken up in 10 cc of isoamyl alcohol.

Four volumes of benzene were added to the isoamyl alcohol extracts and the basic acridines were extracted with two 5-cc portions of 0.5 N hydrochloric acid. Sodium hydroxide was added to the aqueous extract and the strongly alkaline solution was extracted with an equal volume of isoamyl alcohol. After centrifuging, the alcoholic extract was dried briefly with anhydrous sodium sulfate.

For determinations in blood, the alcoholic extract was read directly in the Pfalz and Bauer fluorophotometer. The instrument was equipped with the Corning filter, red ultra No. 584, across the incident beam, and between the fluorescent solution and the photocell a yellow filter (Corning noviol No. 38) and a blue-green filter (Corning No. 553) were used to permit maximum transmission of the yellow-green fluorescence. Concentrations of atabrine between 0.1 and 1.0 γ per cc can be measured within the limits of instrumental error. For all other determinations, one drop of 6 N hydrochloric acid was added

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[1] Hecht, G., *Arch. Exp. Path. Pharmak.*, 1936, **183**, 87.

TABLE I.
Blood Levels of Atabrine Given in mg %.

Rats					Dogs			
Time after dosing, hr	Group 1	Group 2	Days	Group 3	Time after dosing hr	No. 221	No. 97	No. 111
Control	.0	.0	Control	.0	¼	.15	—	—
½	.1	1.3	1	.0	½	.11	.16	.26
1	.1	.5	2	.1	1	.23	.18	.34
2	.3	.8	3	.1	2	.12	.22	.32
3	.6	.2	4	.15	5	.04	.10	.34
4	.4	.4	5*	.1	24	—	.13	.50
6	.4	.3	6	.2				
24	.3	—	7	.15				
			8	.15				

2 rats were used at each interval in Groups 1 and 2, and 4 rats were used for each analysis in Group 3. Average figures are given.

* Daily administration of the drug stopped.

to the alcoholic extract and the color was measured in the Evelyn colorimeter equipped with a No. 420 filter. Concentrations of atabrine between 1 and 10 γ per cc can be measured within instrumental error.

Urine and most tissues gave recoveries of 92% (± 3, maximum deviation 6%). Results were therefore multiplied by the factor 1.09. Liver and spleen gave slightly high values when the atabrine concentration fell below 0.1 mg per gram of tissue. This error was eliminated by using extracts of normal tissue for the instrument blank. Recoveries from blood were 76.5% (±5, maximum deviation 10%). Final values were multiplied by 1.3. Recoveries from feces were 80% (±6, maximum deviation 10%). Final values were multiplied by 1.25. Weise[2] encountered similar difficulties in obtaining complete extraction of atabrine from feces.

Atabrine is detoxicated differently in different animal species, and as many as 4 or 5 isoamyl alcohol-soluble excretion products may occur in the urine of experimental animals.[3] All known detoxication products are extracted by the procedures described above. Such products may occur in blood and tissues, and since we have not attempted to separate these products in the present study, the concentration of atabrine recorded in subsequent tabulations must be regarded as "total" concentrations, including those acridines and related products which occur in the animal organism as the result of atabrine administration.

Animal Experiments. To determine the blood levels of atabrine in the rat following single or multiple doses of the drug, 3 groups of rats were used: 14 rats were given a single, 90 mg per kg dose of the drug; another 14 rats were given a single 225 mg per kg dose; and 32 rats were given 5 daily doses (45 mg per kg). The rats, maintained on a stock diet and water, were deprived of food for 12 hours prior to the administration of the drug by stomach tube. Animals were sacrificed and blood samples were analyzed at the time intervals shown in Table I. The results indicate that large single doses of the drug produce very low blood concentrations, but that these levels remain quite constant over a 24-hour period. The 5 daily doses produced a concentration of only 0.1 to 0.2 mg %, and this level was reached only after 24 hours. This level was maintained rather steadily for at least 3 days after the discontinuation of the drug.

To determine the blood levels of atabrine in the dog following the oral and intravenous administration of the drug, 3 animals were used as follows: Dog No. 221, a male, weighing 5.9 kg, was given 12.5 mg of the drug per kg body weight intravenously over a 2-minute period in the form of a 3% aqueous solution. Dogs Nos. 97 and 111 were followed for a 24-hour period in the course of a study in

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[2] Weise, W., *Arch. Schiffs. Tropen. Hyg.*, 1937, **41**, 715.

[3] Scudi, J. V., and Jelinek, V. C., *J. Biol. Chem.*, in press.

TABLE II.
Elimination of Atabrine from Liver and Spleen of the Rat.

Days after last dose of Atabrine	Group 1		Group 2		Group 3	
	Liver	Spleen	Liver	Spleen	Liver	Spleen
1	1.2	.2	*C 6.2	2.9	2.0	2.5
	0.6	.15	*N 2.9			
	0.9	.2	C 4.4	4.2	C 4.5	2.5
			N 1.5		N 1.5	
3	1.5	.3				
	0.8	.4	—	—	—	—
	1.5	.5				
6	—	—	2.2	1.2	—	—
			3.4	1.2		
			2.2	0.8		
8	0.3	.2				
	0.2	.3				
	0.3	.2				
13-15	0.5	.2	C 1.1	0.9	C 2.0	1.8
	0.7	.1	N 1.2		N 1.1	
	0.2	.1				
	0.1	.2	1.5	1.3	C 2.9	2.2
					N 3.7	

*C = Areas showing congestion with prominent lobular architecture.
*N = Necrotic areas. These were dissected out and analyzed separately.
Data given in mg per g of wet tissue.

which the animals received 50 and 100 mg per kg body weight of the drug by stomach tube per day, respectively. The results are also shown in Table I. In agreement with the findings of Gentzkow,[4] the blood levels were never high. Nor did these levels reflect the dose administered, but the blood concentrations were notably constant.

To study the influence of single and multiple doses of the drug upon the tissue concentrations, and the influence of time upon the elimination of the drug from certain tissues, the following experiments were performed: Group 1—Each of 15 rats were given a single dose of the drug (225 mg per kg body weight) by stomach tube. At intervals of 1, 3, 8, and 15 days after the administration of the drug groups of 3 rats were sacrificed and the livers and spleens were analyzed. Group 2—Rats receiving 12 daily doses of the drug (45 mg per kg) were sacrificed 1, 6, and 13 days after the last dose. Group 3—Rats receiving 12 larger daily doses of the drug (90 mg per kg) were sacrificed 1 and 13 days after the last dose of the drug. In Group 1 of this series the results, shown in Table II, indicate that the concentration of the drug in the liver was not greatly changed 1 to 3 days after the administration of the drug. The concentration dropped appreciably after 8 days but the livers still retained large amounts of the drug 13 days after the last dose. The concentration in the spleen was not greatly lowered even after 15 days. At autopsy the livers and spleens of these animals of Group 1 appeared normal on gross examination, but the animals which received 12 daily doses of the drug (Groups 2 and 3) showed badly mottled livers.

The urinary excretion of atabrine in the rat was studied with the above groups of animals. The animals were housed in metabolism cages in groups of 3 with access to food and water. Urine samples were collected over the time intervals shown in Table III. Each figure represents the analysis of pooled urine from a group of 3 rats.

The data (Table III) indicate that a single dose as large as 225 mg per kg results in a very small urinary output (about 0.25 mg per

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[4] Gentzkow, C. J., *Am. J. Trop. Med.*, 1938, **18**, 149.

TABLE III.
Urinary Excretion of Atabrine in the Rat.

Group 1			Group 2	Group 3
Days after dosing	Atabrine output	Time of urine collection	Atabrine output	Atabrine output
1	.45	First 3 days pooled	0.6	1.2
	.59		0.6	1.2
	.75		0.7	0.9
	.62			
	.99	Second 3 ” ”	1.2	1.3
			1.2	1.5
3	.98		0.8	1.2
	.66			
	.41	Third 3 ” ”	0.9	1.0
	.84		1.1	1.4
			1.0	1.5
8	.43	Drug administration stopped		
	.47			
	.42			
13	.24	3 days after last dose. 24 hr urine	0.5	0.5
			0.4	
		10 ” ” ” ” ” ” ”	0.3	0.5

Group 1: Animals received a single dose of 225 mg per kg body weight.

Groups 2 and 3: Animals received 12 daily doses of 45 and 90 mg per kg respectively. The data are given in mg of atabrine per day.

TABLE IV.
Tissue, Urinary, and Fecal Concentrations of Atabrine.

Tissue analyses (mg per g wet tissue)				Urine and fecal analyses in the dog (mg per 24-hour sample)					
Organ	Monkey	Dog No. 111	Dog No. 104	Days	No. 94 (Feces)	No. 248 (Urine)	No. 247 (Urine)	No. 246 (Urine)	No. 104 (Urine)
Liver	1.4	4.9	0.09	1	†	10	29.5	27.0	25 (30)
Kidney	0.23	1.2*	0.02*	2			12	21.5	16
Small intestinal wall	0.10	1.1		3	18	16	8.5	3.1	16
Brain	0.10	0.07		4	16†				
Thigh muscle	0.30			5	†	6			
Lung	0.30			6	†				
Red Marrow Femur			0.12	7		2.9	2.6	2.5 (18.5)	2.7
Spleen			0.10	8	22		2.4 (2.3)		4.4
				9	30				
				10	31		1.9		
				16			0.3	0.7	0.5

* Equal concentrations were found in the cortex and the medulla.

† 200 mg of atabrine per kg was administered to this dog on these days.

Figures in parentheses are the fecal atabrine output.

rat per day), and that this output does not drop significantly within 3 days after discontinuing the drug. While the urinary output drops after 8 days, the decrement is not large and the drug continues to be excreted for at least 13 days after administration of the drug. The daily urinary output following this single dose is not larger than the output following 3 daily doses totaling only 135 mg per kg. This aberrant elimination of the drug is also evident in the comparison of Groups 2 and 3 where it can be seen that 90 mg of the drug per kg per day does not produce a significantly greater output than 45 mg per kg per day. Thus, the urinary output of the drug does not parallel the dose administered, but tends to reach comparatively low levels. Although these levels diminish with time, as previously reported,[5,6] the drug continues to be excreted by way of the urinary system long after the administration of the drug has been stopped.

Other animals were used to study the distribution of the drug in the animal organism: a monkey which had been given 7 massive doses of atabrine (300 mg per kg body weight) by stomach tube over a 3-week period, Dog No. 111, which received 100 mg of the drug by stomach tube per day, and Dog No. 104, which received an intravenous infusion of the drug (100 mg per kg), were sacrificed and the tissues were analyzed. The data are shown in Table IV. To investigate the localization of the drug in bone marrow, each of 3 rabbits were given a single dose (225 mg per kg body weight) and 6 days later red marrow taken from the femur contained 0.55, 0.71, and 0.79 mg of atabrine per gram of wet tissue. Relatively large concentrations of the drug are found in organs of the reticulo-endothelial system.

The excretion of the drug by the dog was studied as follows: Dog No. 94, given four 200 mg doses of the drug over a period of 1 week, was used to study the fecal output of the drug. Since the dog vomits after the oral administration of large experimental doses of the drug, collection of the fecal samples was arranged on alternate days to avoid contamination with vomitus. Dogs numbered 248, 247, 246, and 104 received a single intravenous infusion of the drug equivalent to 50, 75, 100, and 100 mg per kg body weight, respectively. The urinary and fecal output of this single dose of the drug was then studied over an extended period. All samples taken were analyzed at the time intervals shown in Table IV.

The data shown in Table IV indicate that following oral or intravenous administration of the drug, the urinary excretion of the drug is slow, and urinary concentrations are never high. As previously shown,[5] the drug is excreted by way of the bowel. Relatively large amounts of the drug are excreted in the bowel and this excretion continues for more than a week after the intravenous administration of single large doses of the drug.

Summary. The distribution and excretion of atabrine is similar in the rat and dog. The highest concentrations of the drug were found in the liver. These concentrations were not diminished 3 days after the administration of the drug and although the concentrations were diminished after 8 days, appreciable concentrations were found in the livers as late as 2 weeks after the administration of single or multiple doses of the drug. Intermediate concentrations were found in the spleen and kidney. These concentrations were not significantly diminished 2 weeks after administration of the drug was ceased. Intermediate concentrations of the drug were found in bone marrow and the wall of the small intestine. All other tissues studied showed low concentrations of the drug.

The elimination of the drug is slow. The urinary concentration of the drug never reaches high values, nor does the total output per day reflect the size of the dose administered. Large amounts of the drug are excreted by way of the bowel, and this mode of excretion continues long after the administration of the drug has been discontinued.

Following oral or intravenous administration of atabrine the concentration of the drug in the blood stream remains very low, never exceeding 1 mg % even after the administration of large multiple doses, but these low concentrations are maintained for days after administration of the drug is stopped. The blood concentration bears no apparent relationship to the dose administered. These results together with findings on the distribution and excretion of the drug, suggest that the drug is deposited in the tissues and is slowly liberated, thereby maintaining a rather constant blood concentration of the drug.

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[5] Tropp, C., and Weise, W., *Arch. Exp. Path. Pharmak.*, 1933, **170**, 339.

[6] Chopra, R. N., and Roy, A. C., *Ind. J. Med. Res.*, 1938, **25**, 455.

14333

Effect of Atabrine on the Urinary Porphyrin Output in the Rat.

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In view of the hepatoxic action of atabrine,[1] and the prolonged retention of this drug in the liver, bone marrow, kidneys, and spleen,[2] it seemed desirable to investigate the effect of the administration of atabrine upon porphyrin excretion.[3] In the present study, the rat was used as the experimental animal.

Experimental. We are indebted to Dr. Konrad Dobriner for a crystalline sample of coproporphyrin I-tetramethyl ester. This material in 5% hydrochloric acid solution, when examined in the Beckman spectrophotometer, showed a strong band at 4000 Å. Since the extinction at this wave-length is 25 times as great as the values obtained in the visible (Fig. 1), the Coleman spectrophotometer was calibrated at 4000 Å against solutions of this material.* At concentrations of 0.1 to 1.0 γ per cc these solutions obeyed Beer's Law and the concentrations could be measured within an error of ±3%. These solutions appear colorless to the naked eye, but show an intense red fluorescence when exposed to ultra-violet light.

The method of Rimington and Hemmings[5] was followed in detail. The final use of 0.5% hydrochloric acid as recommended by these authors for the separation of protoporphyrin

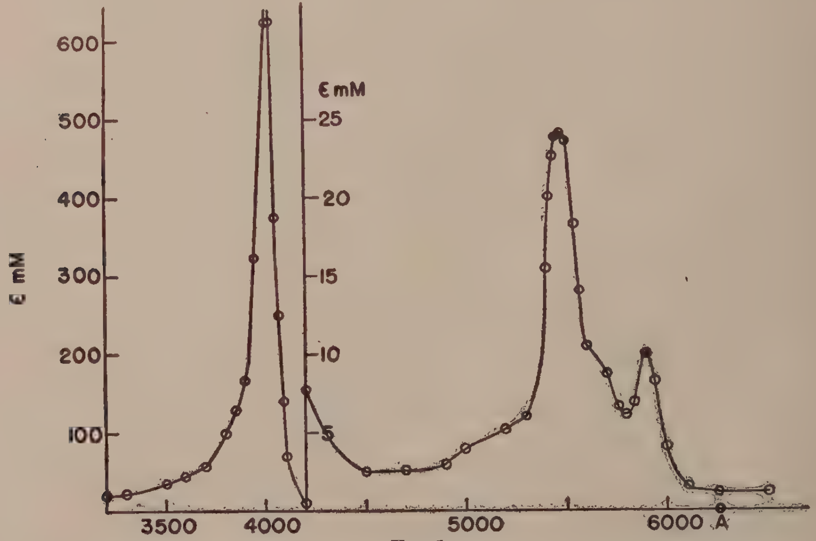

Fig. 1.
The absorption of coproporphyrin I-tetramethyl ester in 5% hydrochloric acid.

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[1] Clark, B. B., Cuminole, B., and Martin, S. J., *J. Pharmacol.*, 1939, **65**, 166.

[2] Scudi, J. V., and Hamlin, M., preceding paper; Hecht, L., *Arch. Exp. Path. Pharmak.*, 1936, **183**, 87.

[3] See Dobriner, K., and Rhoads, C. P., *Physiol. Rev.*, 1940, **20**, 416, for an excellent review of the subject.

* This intense absorption between 3900 and 4100 Å appears to be quite general for the porphyrins.[4]

[4] Fischer, H., and Orth, H., Die Chemie des Pyrrols II, p. 587 *Akad. verlagsgesellsch*, Leipzig, 1934.

[5] Rimington, C., and Hemmings, A. W., *Biochem. J.*, 1939, **33**, 960.

was highly important in the present work since it removed an interfering, green fluorescent material. This material appeared in the urine in increasing amounts as the animals continued on test. The method of Rimington and Hemmings gave excellent results when carried out with pure solutions of the crystalline coproporphyrin and with samples of urine to which 10 and 20 μg quantities of the coproporphyrin were added.

TABLE I.
Urinary Excretion of Atabrine.
Mg per rat per day.

	Dose 5% L.D. 50			Dose 10% L.D. 50		
May '42	1	2	3	4	5	6
7*	.2	.20	.25	.40	.41	.31
10	.41	.33	.29	.43	.50	.40
13	.29	.38	.35	.32	.46	.49
19	.55	.42	—	.65	—	—
26	.20	.30	—	.38	—	—

* Administration of drug begun 5/4/42; ceased 5/15/42. Pooled samples were analyzed, but these values were divided and are expressed in mg per rat per day.

Eighteen white rats, weighing 145 to 190 g each, were placed in metabolism cages in groups of 3, and 72-hour samples of pooled urine were collected. The funnel-shaped lower portions of the cages were coated with paraffin to prevent the urine from coming in contact with the metal. The animals were removed from their cages for 1 hour in the morning and 1 hour in the evening. (A single 2-hour period was used on Sundays). During these periods the animals were allowed free access to water and the stock laboratory diet. During the feeding periods the cages were cleaned. A small amount of urine was unavoidably lost during these feeding periods. Particular care was exercised to prevent fecal contamination of the urine samples.

The animals, maintained under these test conditions for a control period of 9 days, made weight gains of 10 to 42 g indicating that the food intake was sufficient to permit growth. Each of 9 rats was then given 45 mg of atabrine per kilo body weight per day by stomach tube, and the remaining animals were given 90 mg per kilo body weight per day. The experiments were continued for 12 days at which time the animals began to die. During the test period there was a marked loss in body weight.

The pooled samples of urine were analyzed colorimetrically for their atabrine content.[2] The data shown in Table I are given in mg per rat per day. Because of deaths it was not possible to maintain the original groups of rats, but by using the surviving animals individually it was shown, in agreement with previous work,[6] that the drug was excreted for long periods of time after its administration was stopped.

All animals were autopsied as soon as they were found dead. On gross examination all livers appeared severely damaged. Chemical analyses of these livers showed concentrations of atabrine averaging about 3.5 mg per gram of wet tissue with a maximum concentration of 6.5 mg per gram. The spleens showed concentrations averaging about 3.0 mg per gram, with a maximum concentration of 4.2 mg per gram. Three of the 7 surviving animals were sacrificed one week after the administration of the drug was stopped, and the remaining four animals were sacrificed an additional 5 days later. Analysis of tissues from these animals showed about 2.0 mg of atabrine per gram of wet liver, and concentrations in the spleen were about 1.0 mg per gram. Thus, these tissues retain large amounts of the drug for long periods of time.

TABLE II.
Urinary Coproporphyrin Output.

	5% L.D. 50			10% L.D. 50		
Date	1	2	3	4	5	6
4/8/42	17	32	38	12	27	16
5/1	13	28	—	12	21	15
4*	21	27	27	16	31	16
7	32	36	39	24	48	26
10	28	30	34	11	39	14
13	8	18	16	4	15	6

*Drug begun 5/4/42.

The coproporphyrin output by these animals is given in Table II. The values are given in micrograms per group of 3 rats per 3-day collection period. Although liver damage was produced by the massive doses of atabrine used, and high concentrations of the drug were found in the liver and spleen, the data indicate that the urinary coproporphyrin output is not increased by the administration of atabrine. It may be noted that, in comparable experiments with the sulfonamides, Rimington and Hemmings[5] were able to demonstrate a marked porphyrinuria.

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[6] Tropp, C., and Weise, W., *Arch. Exp. Path. u. Pharm.*, 1933, **170**, 339.

Conclusion. The absorption of acidic solutions of the coproporphyrins at 4000 Å is 25 times that observed at 5480 Å, suggesting that measurements should be made at the former wavelength.

Daily oral administration of toxic doses of atabrine does not produce an increased urinary coproporphyrin output in the rat.

14334 P

Effects of Exposure to Oxygen at High Barometric Pressure on Higher Functions of the C.N.S.*

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Observations have been previously made by one of us (J. W. B.) that intermittent exposure of rats to high oxygen pressure not only causes motor dysfunction but also what appears to be a disruption of some of the higher functional activities of the C.N.S. and that these alterations persist for considerable periods after the animal's return to the normal atmosphere. This suggested that less severe exposures to increased O_2 pressure might appreciably affect certain mental processes without causing any gross manifestations in motor function. Experiments were therefore carried out to determine what effect, if any, successive short exposures to O_2 at increased pressure made over a period of several days might have on learning and memory in rats.

The pressure chamber employed was essentially that described earlier (Bean[1]). A continuous circulation of O_2 and removal of CO_2 was maintained by means of a power blower and a canister of soda lime. The O_2 pressure used was 65 pounds (gauge); the temperature was held at 25°C. The experimental animals selected were young adult rats; throughout their exposures to high O_2 pressure they were continuously under the observation of the operator stationed outside the chamber. Convulsive seizures were avoided as far as possible by shortness of the exposures (about 15 minutes) and decompression was carried out so as to insure freedom from the formation of oxygen emboli. Learning and memory tests were made with the Lashley Maze III (Lashley[2]). In the preliminary trials it was found that the O_2 exposures so affected some of the rats that they frequently failed to run the maze for several hours after their removal from the pressure chamber. For this reason an interval of one day was provided between the removal of the animals from the chamber and their subsequent test in the maze.

Learning. The facility shown in learning the maze was determined by two measures, *viz.*, the number of trials, and the number of errors, made before reaching the learning criterion. No significant difference was found in either respect between those animals which had been exposed to increased O_2 pressure and in the control animals which had been kept at normal atmospheric conditions.

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* The authors are indebted to the Horace Rackham School of Graduate Studies, University of Michigan, for partial support of this investigation, and to Dr. N. R. F. Maier for invaluable advice, criticism, and his cooperation in placing at their disposal the maze facilities of the Psychological Laboratory for the Study of Abnormal Behavior in the Rat.

[1] Bean, J. W., PROC. SOC. EXP. BIOL. AND MED., 1929, **26**, 832.

[2] Lashley, Karl Spencer, *Brain Mechanisms and Intelligence*, Chicago, University of Chicago Press, 1929.

Memory and Retention. To test for memory and retention a group of animals learned the maze and was then divided into two matched groups, experimental and control. The experimental group was subjected to increased O_2 pressure in 16 successive exposures over a period of 10 days. The control group was treated in the same way except for the exposure to oxygen. One day after the last oxygen exposure of the experimental group, both it and the control group, were tested for maze retention. It was found that the experimental group made an average of 52.9 errors in relearning the maze, whereas the control group made on average of only 2.7 errors. This difference is strikingly significant. Thus the memory and retention of the maze previously learned was definitely impaired in the experimental group of animals as a result of their exposure to the increased O_2 pressure.

Conclusion. The data were interpreted to mean (1) that successive intermittent exposures to O_2 at high barometric pressure had no effect on the ability to learn a maze habit; (2) that the retention and memory of a maze previously mastered was adversely affected to a striking degree by intermittent exposures to increased O_2 pressure; (3) that certain mental processes may suffer deleterious effects as a result of exposure to increased O_2 pressure even when such exposure causes no evident motor dysfunction.

14335

Similarity of Response of Thymus and Lymph Nodes to Administration of Adrenocorticotropic Hormone in the Rat.*

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Enlargement of the thymus and lymphoid tissues has been noted experimentally and clinically[1] as the result of adrenalectomy or hypofunction of the adrenal cortex. On the other hand, adrenal cortical extracts[2] and the administration of adrenocorticotropic hormone[3] have been shown to cause thymic involution. Since it has been shown experimentally[4] that lymph nodes respond to adrenalectomy in the rat in the same manner as does the thymus, it is of interest to note that administration of purified adrenocorticotropic hormone causes a similar involutionary change in lymph nodes as well as thymus.

Methods. The reaction of thymus and lymph nodes to purified sheep adrenocorticotropic hormone (ACT)[5] was established in the following experiments. (1) Action of ACT in the normal rat. (2) Action of ACT in the hypophysectomized rat. (3) Action of ACT in the adrenalectomized rat maintained in good condition on 1% NaCl. All animals employed were 26-day-old male rats at onset of the experiment. The treated animals were given a total dose, daily, of 1 mg ACT by intraperitoneal injection in 3 injections distributed through the 8-hour-day (2 injections on Saturday, 1 injection on Sunday). Injections were begun on the day of operation. All animals were maintained under identical nutritional and environmental con-

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* Aided by grants from the Board of Research of the University of California and the Rockefeller Foundation of New York City.

[1] Moon, V. H., *Shock*, Lea and Febiger, Philadelphia, 1942, 198-203.

[2] Ingle, D. J., Higgins, G. M., and Kendall, E. C., *Anat. Rec.*, 1938, **71**, 363.

[3] Evans, H. M., Moon, H. D., Simpson, M. E., and Lyons, W. R., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 419.

[4] Reinhardt, W. O., and Holmes, R. O., *Ibid.*, 1940, **45**, 267.

[5] Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, 1942, **96**, 450.

TABLE I.
Effect of Adrenocorticotropic Hormone on Weights of Thymus and Lymph Nodes.

Type of rat	No. of rats	Final body wt, g	Thymus wt, mg	Cervical lymph node wt, mg
Unoperated				
Control	6	236 (210-252)†	579 (475-700)	347 (193-423)
Treated*	6	158 (142-175)	72 (39-100)	83 (38-117)
Hypophysectomized				
Control	5	114 (100-120)	219 (98-299)‡	185 (123-289)
Treated	5	107 (95-124)	43 (26- 52)	99 (60-114)
Adrenalectomized				
Control	10	186 (147-224)	550 (389-760)	400 (308-505)
Treated	10	188 (152-227)	549 (335-750)	385 (255-502)

* 1 mg daily dose divided in 3 injections during an 8-hr period; Saturday, 2 injections, and Sunday, 1 injection. Injections were begun on the day of operation. Experimental periods were of 30 days duration with the exception of the hypophysectomized group which were treated for 15 days. All rats were males, 26 days of age at onset of the experiment.

† Ranges in the group.

‡ 4 animals.

ditions. Duration of the experiment was 30 days for the normal and adrenalectomized animals, 15 days for the hypophysectomized group. At autopsy, thymus and cervical lymph nodes were removed according to a technic previously described,[4] weighed and fixed for histological study.

Results. The results of the above experiments are found in Table I. It is apparent that ACT produces a striking and similar effect on the weights of the thymus and cervical lymph nodes. That the remarkable reduction in the size of these tissues is not due to reduction in food intake has been ruled out by other experiments in which (1) food intake of the control and injected animals was measured and found to be identical, and (2) food intake of controls was restricted to the point where the control animals maintained the same body weight as ACT injected animals; in these experiments the thymus and lymph nodes underwent some reduction in weight, but this reduction was not in any sense as extensive as that produced by ACT injection. Effects were noted after ACT was injected into either immature (26-day-old) or mature (89-day-old) male rats. Histological examination of the lymph nodes of the ACT-treated rats shows a flushing out of the adult lymphocytes with a corresponding decrease in the size of the other components of the node. There was no histologically observable effect of the ACT injections on the nodes of the adrenalectomized animals.

It seems apparent that ACT acting through the adrenal cortex is effective in producing a striking reduction in weight and size of the thymus and cervical lymph nodes in the rat. This confirms the recent similar findings of Dougherty and White for the mouse.[6] It is probable that this effect is not mediated by the pituitary gland, since the effect is easily demonstrable in the hypophysectomized animal. It is further doubtful that the effect is mediated through the gonads, since it has been shown elsewhere[3] that thymus reduction after ACT injection occurs with equal facility following double ovariectomy. It is likewise doubtful that secretion of an androgenic substance by the adrenal cortex under the influence of ACT treatment is responsible for the effects on the thymus and lymph nodes, since in none of the experiments here cited was there evidence of coincident stimulation of the accessory sexual tissues (prostate, seminal vesicles, Cowper's gland).

These experiments furnish evidence that there is a reciprocal relationship as regards the effect on the thymus and cervical lymph nodes of adrenalectomy on the one hand, and administration of ACT on the other. It is notable that these tissues show probably a more profound reaction to these procedures than do any other tissues in the body. Certainly this reaction is of some importance to the animal economy. Further experimental procedures should give some clue to the function of this widespread organ system and to the fundamental importance of the lymphocyte.

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[6] Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 132.

Summary. Administration of purified ACT to the male rat produces by its action on the adrenal cortex a striking reduction in the weight and size of the thymus gland and the cervical lymph nodes. This same effect is not manifest in the adrenalectomized animal. This finding furnishes further evidence for the existence of a definite relationship between adrenal cortex and thymus and lymph nodes. Functional interpretation of this relationship awaits further experimental and clinical study.

14336

Age as a Factor in Susceptibility of Mice to the Endotoxin of Bacillary Dysentery.

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As a part of a series of studies dealing with the effects of bacterial toxins on mice,[1-5] experiments were set up to ascertain the susceptibility exhibited by mice of different ages, and therefore, of different body weights, to a specific endotoxin. The toxic material used in these experiments was obtained from the cell bodies of *Shigella paradysenteriæ* Flexner by methods previously described.[5]

Three age-weight groups of normal male Rockland mice were chosen for the tests, and comprised 12-, 20-, and 35-g mice with ages of approximately 25, 50, and 350 days, respectively.* Survival curves were obtained by injecting the endotoxin in dosages ranging from the minimal effective killing level to that causing death in nearly all the mice. All injections were of aqueous solutions by the intraperitoneal route. Survivors were counted at the end of a 24-hour interval, experience indicating this to be an adequate period. The data from two duplicate experiments are presented graphically.

In Fig. 1 the experimental data are presented as percent survival against dosage expressed in the usual terms of grams of toxin per kilogram of body weight. The curves indicate different susceptibilities for animals of the 3 age-weight groups. This difference may be interpreted as meaning that the 35-g mice, at the 50% survival level, are about 3 times more sensitive to the lethal action of the toxin than are the 10-g mice, and somewhat less than twice as sensitive as the 20-g. It would be assumed from such an interpretation that the aging process in mice involves an increasing sensitivity to the endotoxin.

A more plausible interpretation of this type of toxication becomes apparent if percent survival is plotted against the absolute dose required to kill (Fig. 2). The curves obtained when the data are plotted in this manner appear to be almost superimposable within the limits of experimental error. This suggests that the toxic material in causing death in the mouse acts by disabling a single physiological system, and that this unit injury is to a system which is substantially independent of age or normal body weight for the range tested.

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[1] Zahl, Paul A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224.

[2] Zahl, Paul A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 285.

[3] Zahl, Paul A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 116.

[4] Hutner, S. H., and Zahl, Paul A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 364.

[5] Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, in press.

* The mice used in these experiments were of a standardized inbred strain tested at regular intervals for freedom from bacterial infections.

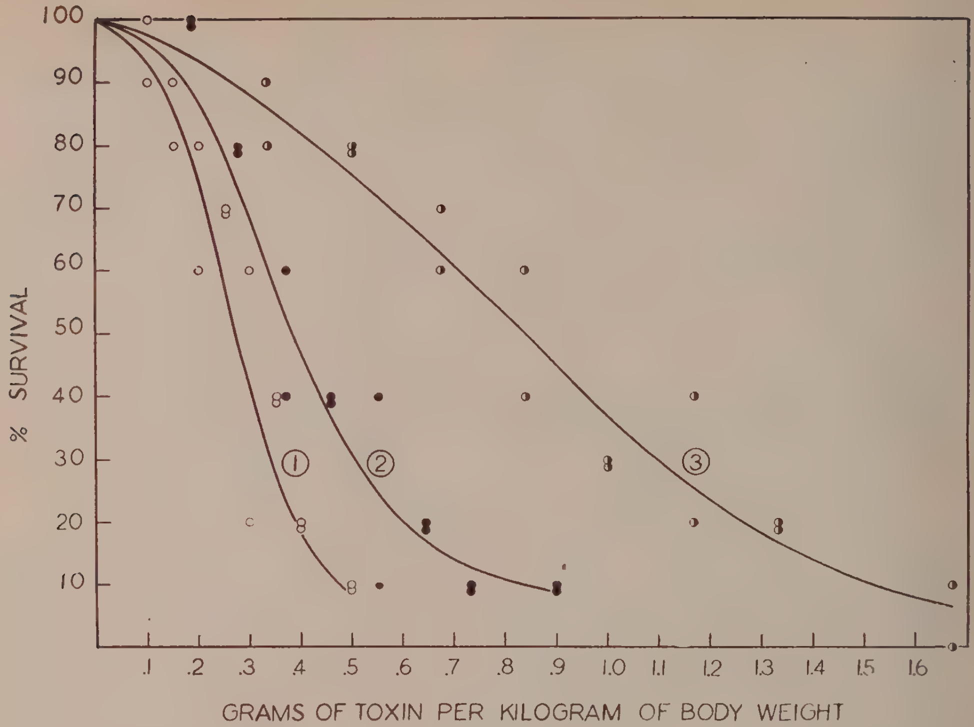

FIG. 1.

Survival curves of mice receiving intraperitoneal injections of *Shigella* endotoxin, computed on the basis of grams of dry toxic material per kilogram of body weight. (1) 35 g mice; (2) 20 g mice; (3) 12 g mice. Each point plotted refers to 10 animals.

It appears probable that the unit injury in this case is direct vascular damage. This view is supported by the current belief that the somatic O antigens of gram-negative bacteria exert their toxicity primarily on the vascular system, particularly the capillary endothelium.[6-11] Dennis presents this point of view perhaps most clearly in generalizing from his studies on the typhoid endotoxin: "The purified somatic antigen complex is a vascular poison. The toxic manifestations and histopathology are entirely attributable to extensive capillary hemorrhage."

The toxicity of the preparation used here is referable in almost full measure to the somatic O antigen, the strain being smooth and virulent for mice. That this kind of vascular damage is characteristic of the endotoxins of gram-negative bacteria has been demonstrated by the uniform production of hemorrhage in transplanted tumors following the injection of the endotoxins of almost any gram-negative bacterium.[1,4] This property is lacking in gram-positive bacteria, which do not possess chemically similar somatic antigens. One would suppose from the uniform pattern of vascular damage produced by gram-negative endotoxins that the results reported here for *Shigella* would be obtained with the endotoxins of other gram-negative bacteria, whether

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[6] Cameron, G. R., Delafield, M. E., and Wilson, J., *J. Path. and Bact.*, 1940, **51**, 223.

[7] Dennis, E. W., *Proc. Third Internat. Cong. Microbiol.*, New York, 1940, 797.

[8] Kourilsky, R., Kourilsky, S., and Bablet, J., *C. R. Soc. Biol.*, 1941, **135**, 18.

[9] Penner, A., and Bernheim, A. I., *J. Exp. Med.*, 1942, **76**, 271.

[10] Morgan, Herbert R., *Am. J. Path.*, 1943, **19**, 135.

[11] Olitzki, L., Bendersky, J., and Koch, P. K., *J. Immunol.*, 1943, **46**, 71.

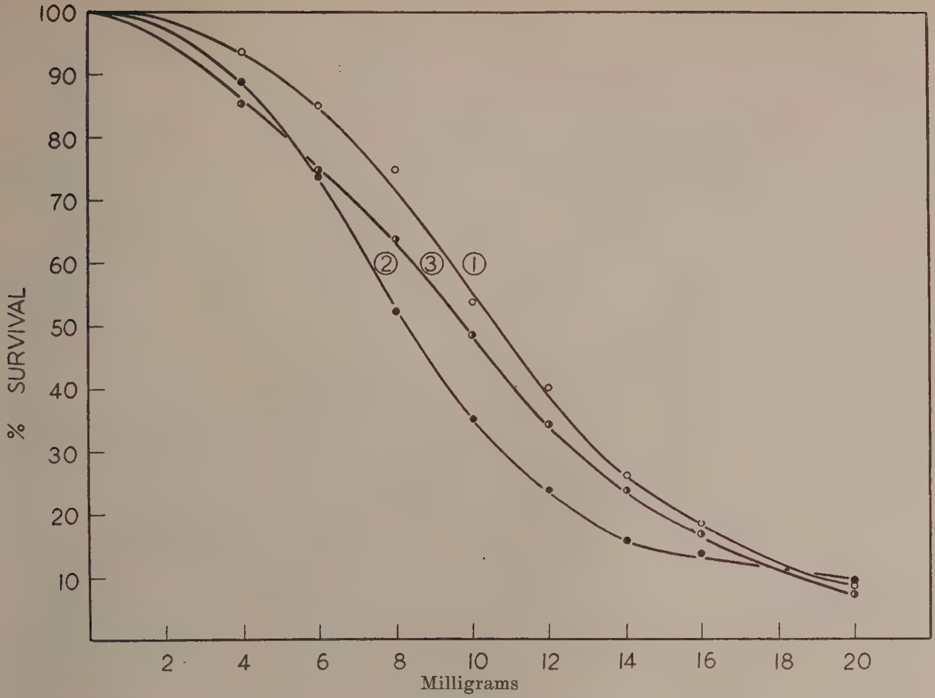

FIG. 2.

Survival curves of mice receiving intraperitoneal injections of *Shigella* endotoxin, computed on the basis of absolute amounts of dry toxic material injected. (1) 35 g mice; (2) 20 g mice; (3) 12 g mice.

the endotoxins were administered as bacterial extracts or liberated in the course of an infection by living bacteria.

The range of weights and ages covered by our data does not include the extreme limits of the life cycle where vascular susceptibilities might be expected to differ markedly.

It seems profitless to attempt a comparison of this type of toxication with those reported in the literature dealing with the results of administration of organic and inorganic poisons. In a number of studies mature rats have been described to be more sensitive than young rats to certain poisons, and equally or less sensitive to others.[12] Such results make to appear almost certain that different toxication mechanisms are involved.

Summary. Dose-survival curves obtained by injecting the endotoxin of *Shigella paradysenteriæ* Flexner into 12-, 20-, and 35-g mice indicate that such mice are killed by approximately the same minimal amounts of toxin, and that this effect is not considerably modified within the limits of age or normal weight indicated. This effect is interpreted as injury to a single system, most probably the vascular system.

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[12] Holck, H. G. O., *The Rat in Laboratory Investigation*, J. B. Lippincott Co., Philadelphia, Pa., 1942, p. 302.

14337

Toxicity of Extracts of Derris Root for Mice.

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Several authors[1,2] have shown that the toxicity of derris for mammals does not necessarily parallel its rotenone content or its content of other known active compounds or fractions. It is conceivable that this is due to the presence in the root of an as yet unidentified fraction. During efforts to clarify this situation we have made certain observations on the toxicity of several derris extracts, which data we feel are worthy of reporting, although they do not answer the main question.

Experimental. A sample of finely ground derris root containing 4.6% rotenone and 14.0% total chloroform extractives was used. The different fractions were prepared by extraction with organic solvents in a Soxhlet, or by shaking a suspension with water.[3] The extracts from the organic solvents were deposited on talc by evaporation of the solvent, but the colloidal water extract was used as such. The two samples of talc, of different particulate size, were prepared by fractionation in an air classifier. The rotenone, chloroform extractive, and red color values (rotenone plus deguelin),[4] together with a description of all samples, are given in Table I.

The acute oral $L.D._{50}$ toxicities of the sample of derris and its several fractions were determined on white mice of mixed sexes, weighing from 16 to 22 g. The materials were used in water suspension. After preliminary trials 20 or more mice were used at each of from 3- to 5-dose levels, and the $L.D._{50}$ was determined from the charted results. The animals were kept under observation for 5 days or longer after administration of the materials.

Discussion of Results. These studies (Table I) show that, with the specimen of derris used, all materials highly toxic to mice were removed by complete extraction with chloroform. This chloroform extract when deposited on talc did not have as great toxicity as it did in the original form in whole derris, probably because of the absence of certain dispersing agents which are present in the root, and which cause colloidal suspensions of the toxicants to be formed when the root is administered alone. This dispersing action is demonstrated by the ease with which colloidal water extracts can be prepared from the root. The water extract was shown to be relatively highly toxic, although water recovers only 30% of the rotenone-deguelin content as determined by the red color test. The marc, still containing 70% of these materials, was only slightly more toxic. The toxicity of the petroleum ether extract and the marc from this extraction was related to the amount of insecticidal substances present as estimated by the red color test. Further extraction with cyclohexane of the marc from the petroleum ether extraction reduced the toxicity to a very low value. Most of the remaining material is probably retained in unbroken plant cells.

Comparison of the same toxic material (petroleum ether extract) deposited on two different samples of talc shows that the more finely divided sample had the higher toxicity. Lightbody and Mathews[5] previously have reported finely powdered rotenone to be more toxic for rats than the crystalline form. We have not been able thus far to isolate an active alkaloid or glycoside from any of the

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[1] Mathews, J. A., and Lightboy, H. D., *Ind. Eng. Chem.*, 1936, **28**, 812.

[2] Ambrose, A. M., and Haag, H. B., *Ind. Eng. Chem.*, 1936, **28**, 815.

[3] Goodhue, L. D., and Haller, H. L., *J. Econ. Entomol.*, 1939, **32**, 877.

[4] Goodhue, L. D., *J. Assn. Off. Agri. Chem.*, 1936, **19**, 118.

[5] Lightboy, H. D., and Mathews, J. A., *Ind. Eng. Chem.*, 1935, **28**, 809.

TABLE I.
Acute Oral Toxicity for White Mice of Derris Root and Various Extracts of It.

Sample	Rotenone, %	Total Chloroform Extractives, %	Red color % of total	$L.D._{50}$ white mice, mg/kg
1. Powdered derris root	4.6	14.0	100.00	350
2. Marc, after extraction of No. 1 with water			70.5	470
3. Marc, after extracting No. 1 with petroleum ether 20 hr	2.44	6.8	50.7	570
4. Water extract of No. 1			31.0	600
5. Petroleum ether extract of No. 1 on talc having mean diameter of 1 μ	2.20	7.2	56.0	600
6. Petroleum ether extract of No. 1 on talc having mean diameter of 2 μ	2.20	7.2	55.0	700
7. Total chloroform extract of No. 1 on 2 μ talc	4.6	14.0		700
8. Chloroform extract of No. 3 on 2 μ talc	2.4	6.8	44.0	1,100
9. Marc after extracting No. 3 with water			40.5	3,000
10. Marc from complete extraction of No. 1 with chloroform	0	0	0.0	>3,600*
11. Marc from No. 3 extracted 20 hr with cyclohexane	1.5	4.5	20.0	>1,200†
12. Talc control, 2 μ				>4,200*

* No fatalities. † 10% fatalities.

fractions, or from whole derris.

Summary. From studies dealing with the acute toxicity of derris and several extracts thereof for mice, it appears that this toxicity is due to the insecticidally active materials represented by the rotenone content, total chloroform extractives content, and red color index. No single one of these three determinations, however, seemed uniformly to indicate the relative toxicity of the various fractions studied. Materials not extractable with chloroform were found to be practically nontoxic. Thus far no active alkaloid or glycoside has been isolated from derris.

14338

Changes in the Adrenals Following Prolonged Treatment with Methyl-Testosterone.

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The fact that adrenal cortical tumors are often accompanied by clinical signs of "virilism" called attention, at an early date, to possible relationships between the adrenal cortex and the production of testicular or "testoid" hormones. It has been found that the zona reticularis or "x-zone" of the adrenal cortex in the mouse undergoes rapid involution under the influence of testoid hormones.[1–3] In other species, however, this is not the case. For instance in the rat testosterone and its propionate fail to elicit specific changes in the reticularis although they decrease the weight of the organ due to a slight involution of the cortex as a whole.[4,5] It has been especially emphasized that in the rat testosterone treatment during the first few weeks of life causes a particularly pronounced involution of the glomerulosa without eliciting any significant change in the reticularis.[6] In acute experiments on rats Nathanson and Brues[7] found, on the other hand, that testosterone propionate stimulates mitotic activity especially in the glomerulosa as judged by the colchicine technic.

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* Working under a grant from the Banting Research Foundation.

[1] Howard, E., *Am. J. Physiol.*, 1940, **129**, P 297.

[2] Burrows, H., *J. Path. and Bact.*, 1936, **43**, 121.

[3] Starkey, W. F., and Schmidt, E. C. H., Jr., *Endocrinology*, 1938, **23**, 339.

[4] McEuen, C. S., Selye, H., and Collip, J. B., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 390.

[5] Selye, H., *Canad. M. Assn. J.*, 1940, **42**, 113.

The object of this communication is to report upon a series of experiments which indicate that the rat adrenal responds just as specifically to chronic overdosage with testoids as that of the mouse, although the type of the resulting morphological changes is entirely different.

Twelve male albino rats weighing 60-75 g (average 68 g) were subdivided into 2 groups of six. The animals of the first group received 2 daily injections of 0.1 cc of peanut oil containing 5 mg of methyl-testosterone in the form of finely ground crystals in suspension. The remaining 6 rats were treated with cholesterol in the same manner and dosage, since cholesterol is devoid of any hormonal activity they served as controls. The animals were killed 76 days after initiation of treatment and their adrenals were weighed after fixation in Suza mixture. The average weight of the adrenals in the methyl-testosterone treated group was 32 mg as compared with 38 mg in the controls, a difference which was not statistically significant ($P = 0.1$).

Histological examination of these adrenals, on the other hand, showed very characteristic changes. The glomerulosa appeared narrower than that of the controls and consisted of small cells which were very poor in cytoplasm. The connective tissue capsule of the gland was greatly thickened. The other layers of the cortex did not participate in the atrophy but likewise showed typical alterations. Throughout the cortex a large number of epithelial cells was filled with voluminous vacuoles. In frozen sections stained with Sudan III these proved to contain lipid material. In many cases a single large lipid granule distended the cell body and pushed the nucleus to one side so that "signet ring" types resulted resembling the fat cells of ordinary adipose tissue (Fig. 1 and 2). Fat-laden cells are usually also

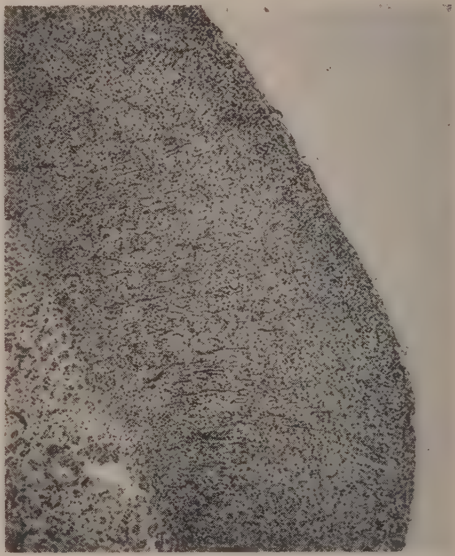

FIG. 1.

Normal adrenal cortex of control rat.

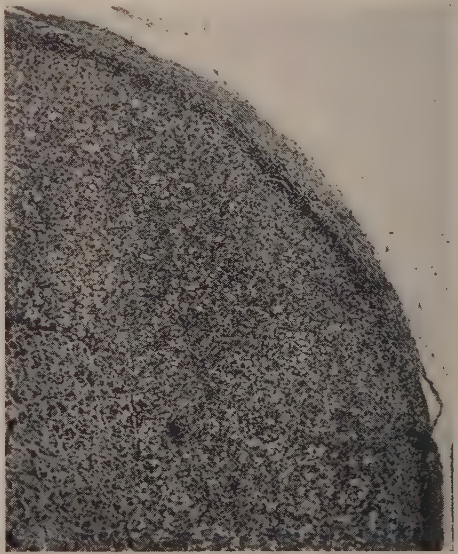

FIG. 2.

Adrenal cortex of rat treated with methyl-testosterone. Note thickening of connective tissue capsule, involution of glomerulosa (dark zone) and vacuoles in remaining cortical cells.

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[6] Selye, H., *Anat. Rec.*, 1940, **76**, 145.

[7] Nathanson, I. T., and Brues, A. M., *Endocrinology*, 1941, **29**, 397.

found in the adrenal medulla of the rat and in the hormone-treated group these medullary cells were likewise characterized by unusually coarse lipid granules. This change was not observed in animals treated with steroids devoid of testoid properties. It will be recalled that folliculoids cause hypertrophy of the cortical cells accompanied by an actual loss of lipid granules while corticoids induce a generalized atrophy of the cells in all 3 layers of the cortex; thus the response of the adrenal to treatment with testoids appears to be rather specific.

The influence of the adrenal cortex on potassium metabolism is well known and in connection with the adrenal changes described above it is of interest to note that Butler *et al.*[8] observed a pronounced fall in the serum potassium of patients treated with methyl-testosterone. We therefore determined the serum potassium concentration (method of Kramer and Tisdall[9] modified by Jacobs and Hoffman[10]) of our rats on several occasions during the course of the experiment. However, in this respect there was no statistically significant difference between the two groups.

It is also of interest to note that in spite of this long continued treatment with high doses of methyl-testosterone, these animals showed no other manifest signs of damage except for a slight inhibition of somatic growth, their average final body weight being 196 g while that of the controls was 236 g.

Summary. Experiments on male albino rats indicate that chronic treatment with large doses of methyl-testosterone elicit changes in the adrenal cortex which are characterized by an involution of the glomerulosa, marked thickening of the connective tissue and deposition of coarse fat granules in the cells of the fasciculata and reticularis. In many cases these fat granules fuse into a single voluminous lipid globule which distends the cytoplasm and pushes the nucleus to one side. Consequently the epithelial cells of the cortex assume the appearance of ordinary fat cells. Although in the rat testoid compounds do not cause a specific involution of the reticularis as they do in the mouse, the above mentioned changes are considered to be a typical response of the cortical cells to overdosage with testoid substances.

Contrary to previous observations in man the serum potassium showed no significant change following treatment with methyl-testosterone in the rat.

The expenses of this investigation were defrayed through a grant received from the DesBergers-Bismol Laboratories. The authors are also indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N.J., who supplied the necessary amount of methyl-testosterone.

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[8] Butler, A. M., Talbot, N. B., and MacLachlan, E. A., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 378.

[9] Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, **46**, 339.

[10] Jacobs, H. R. D., and Hoffman, W. S., *J. Biol. Chem.*, 1931, **93**, 685.

14339 P

Use of Bacterial Asphyxia for Homologous Antigen Production in Experimental Immunization Against Human-Type Tuberculosis.*

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In tuberculosis no wholly satisfactory immunity of man and domestic animals has been obtained. This may be due in part to the unsatisfactory constitution of the vaccines employed. It is therefore restated that human, bovine, and avian type bacilli may be killed

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* Received support from the Seymour Coman Fund.

without the use of coagulating heat or of chemicals, by incubation at 38-40°C in a partial vacuum, saturated with water vapor but deprived of oxygen.[1] In this condition the bacilli are evidently forced to metabolize in the absence of a new supply of one of their most essential constituent elements. Perhaps they are "sabotaged" by their own internal enzymes but oxidation of the protoplasmic substance is inhibited by the absence of molecular oxygen. The death of these bacilli, from the sequelæ of oxygen deprivation in a moist warm state, I have called, for lack of a better term, asphyxia. Various investigators have communicated a scattering of published or unpublished observations suggesting that many, if not all, disease parasites might be killed in a somewhat similar fashion. Some adaptations of the technic to deprive the anaerobes of combined oxygen, to fit the time and temperature of incubation to the delicacy of particular parasites, and to remove exotoxins after killing, appear indicated for other organisms. Yet in general the application of parasitic asphyxia to the needs of immunology appears to be a wide and virtually untried field.

Marked protection against avian tuberculosis of the rabbit, by a vaccine killed in an essentially similar fashion (incubation of glass-sealed cultures), has previously been reported.[1] However, it could not be assumed from these observations on the avian bacillus that the human-type organism would also autoasphyxiate itself without irreparable mischief to its specific immunizing power. Also it seemed desirable to speed up the killing for these tough vegetative parasites by immediate elimination of oxygen and medium from the glass-sealed tubes.

In the present experiments, therefore, pyrex glass tubes each containing 0.5 cc boiled tap water[†] and the growth removed from 10 one-month-old glycerine-agar cultures of human tubercle bacilli (strain H63) were prepared. The tubes were evacuated to a reading of 2 cm pressure, degassed, washed out repeatedly with hydrogen, evacuated, and glass-sealed at about 2 mm residual pressure of water vapor. A few drops of liquid water remained in each tube. The contents were then sterilized by incubation for one month at 40°C. Death of the bacilli was confirmed by attempted culture on Petragnani medium, and by attempted infection of 16 guinea pigs and 8 hamsters.

For immunization, the rabbit was selected as an experimental animal. Large intravenous doses (0.1-0.5 mg) of virulent human strains, in rather coarse suspensions, produce in this animal a largely self-limited disease characterized by extensive morbidity but low mortality.

Litters of 4-6 rabbits each, free from coccidiosis and snuffles, were selected. Each litter was derived from animals inbred, brother and sister, for 3-4 generations. Sixty-six rabbits in all, in 2 series, were employed. One-half of each family was reserved for controls.

The other half of each family was immunized by 6 weekly subcutaneous injections, partly in each quadrant, of approximately 13 mg each of the asphyxiated vaccine. A portion of each thick, milky suspension of the bacilli was tested at the same time for any residual virulence by inoculation in the guinea pigs and hamsters. Four months after immunization all living rabbits were intravenously inoculated with virulent bacilli. Three doses ranging from 0.1-0.5 mg were employed in different groups.

Two control animals and no immunes died prior to test injection with living bacilli, apparently from heat prostration. The immunes, although young 4-lb animals at the start, were but little retarded in weight gain as compared with the controls by the heavy doses (totaling 78 mg) of vaccine they received. After test injection of living bacilli, 4 controls and 3 immunes died at 3-6 months; these deaths could not be definitely ascribed to tuberculosis alone; they were due in part to heat prostration. At 8-10 months the remaining animals were killed in pairs. One whole family of 6 animals inoculated with the smallest dose showed at this time no gross lesions in either controls or immunes. All other controls, except 2, showed gross lesions, often extensive, of the lungs, kidneys, oviducts,

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[1] Potter, T. S., *J. Infect. Dis.*, 1942, **71**, 220, 232; *Fed. Proc.* (Imm.), 1943, **2**, 101.

[†] Phosphate buffer solution may prove a distinct improvement.

TABLE I.

	Controls	Immunized
No. of rabbits at start	33	33
Died before test infection	2	0
Died (in heat wave)	4 (3 showed lesions)	3 (1 showed lesions)
Resistant family showing no lesions	3	3
Others showing no lesions at 8-10 months	2	24
Total showing lesions at 8-10 months	22	3

testes, mammary glands, bones, or subcutaneous lymph nodes. Microscopic sections of these lesions showed frequently fuchsinophile detritus with only a few intact bacilli. The immunes, except 3, totally escaped such lesions; when present lesions were few, and were small ones of the kidneys or subcutaneous lymph nodes.

Comment. The best experimental results on immunization against tuberculosis have been obtained previously by use of living tubercle bacilli heterologous in type, such as BCG,[2] and with heat-killed bacilli, homologous in type.[3] In sharp contrast to the results obtained in immunization with whole bacilli, living or dead, I have found to date no record of successful results with chemically fractionated bacilli. Satisfactory comparison between the living and non-living vaccines is difficult because the living persist longer and doubtless multiply for a time thus producing more numerous and persistent foci of stimulation than so far obtained with killed organisms. For purposes of comparative study the largest well tolerated doses rather than equal doses might well be employed. The killed and non-multiplying vaccines present the advantages of using the very type of parasite one wishes to immunize against, and of superior control of the final quantity, distribution. and persistence of the antigen.

The results here submitted and those previously presented confirm the conclusion of Opie and Freund[3] that a considerable protection of the rabbit is possible by use of completely killed tubercle bacilli as a vaccine. Protection of the guinea pig[‡] has also been observed with asphyxiated bacilli but less complete than that obtained simultaneously with equal doses of BCG, and less than that of the rabbit. As pointed out by Opie and Freund the rabbit diseases resemble human tuberculosis in virulence far more than the highly fatal guinea pig infections. (*Cf.* high incidence of tuberculin reaction in people offering no clinical symptoms, and the low death rate even in sanatoria). Avian swine tuberculosis is also a disease of moderate virulence.

Due to limitations in animal space no conclusive comparative work between heat killed and asphyxiated bacilli has been completed to date. The doses of asphyxiated vaccine used in rabbits were approximately 30 times those of heat-killed vaccine employed by Opie and Freund and 300 times those of BCG clinically advisable. The human test strain of bacilli employed by me was less virulent than the Ravenel bovine strain employed by Opie and Freund, but the test doses were approximately 10,000 times as great and the protection obtained apparently more complete.

Summary and Conclusion. Following the immunization of 33 rabbits with tubercle bacilli killed by asphyxia, 29 of the animals showed (mostly at 8-10 months) no tuberculous lesions. Among 31 normal siblings of the above 25 exhibited gross tuberculous lesions. Thus parasitic asphyxia is a flexible and virtually untried principle for use in immunology. It appears promising for study in human-type as well as in avian-type tuberculosis.

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[2] Townsend, J. G., Aronson, J. D., Saylor, R., and Parr, I., *Am. Rev. Tub.*, 1942, **45**, 41.

[3] Opie, E., and Freund, J., *J. Exp. Med.*, 1937, **66**, 761.

[‡] Low toxicity of the whole asphyxiated bacilli for guinea pigs was demonstrated.

14340

O-Benzoic Sulphinide (Saccharin) Ferridehemoglobin.

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In discussing the reactions of nitrite with blood pigment, Gamgee[1] claimed priority on the discovery of the class of ferrihemoglobin derivatives typified by the cyanmethemoglobin of Kobert.[2] Smith and Wolf[3] discovered the azide and Haurewitz,[4] the fluoride derivatives. On the basis of its potentiometric behavior, Barnard[5] characterized alkaline ferrihemoglobin as the hydroxide of this class and predicted the ability of ferrihemoglobin to form polar valent compounds with many anions. Keilin[6] described the hydrosulfide of ferrihemoglobin and in 1937, Barnard[7] designated the class of derivatives as "hemoferrides" to emphasize their binary nature, *i.e.*, they were salts of hydro-acids, exclusively.

On the basis of the demonstration of the nitrile oxide structure of fulminic acid and the fulminates by Pauling and Hendricks,[8] Barnard and Neitzel[9] prepared a fulminate-ferrihemoglobin compound. Hydrogen selenide derivatives of ferrihemoglobin and ferriheme were also prepared.[10] Combinations between thiocyanate and ferrihemoglobin and between cyanate and ferrihemoglobin were shown to exist by Jung[11] and Hecht[12] respectively. Thiocyanic and cyanic acids are now known to be hydroacids.[13] A cyanamide derivative of ferriheme[14] and ferrihemoglobin[15] have also been prepared. In the description of the cyanamide compounds, the terms ferrideheme (in place of hemoferride), ferridehemochromogen and ferridehemoglobin were introduced in order to adapt to these substances, the system of nomenclature proposed by Pauling and Coryell[16] for the blood pigment derivatives.

It is assumed that the ferridehemoglobin engendering combination between ferrihemoglobin and hydro-acid anions is conditioned by the comparatively small ionic diameter of the latter. Of the relatively limited class of acids which are not oxy-acids, the majority do combine with ferrihemoglobin. Potentiality for this type of combination seems to be restricted to the smaller members of the class; the halides with the exception of the smallest, fluoride, do not so combine. In those instances where the rule interdicting ferrideheme formation with oxyacid anions appears to be excepted, as in the cases of hydroxyl, peroxide, and nitrite ion; the first two have ionic mobilities comparable with those of hydro-acid anions and the last may be coordinated as nitro ion. It has been suggested that the small ionic diameter of hydro-acid anions permits their penetration of the interstices of the porphyrin ring and the formation of polar covalent bonds with the iron.[15]

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* On active duty, Medical Corps, Army of the United States.

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[2] Kobert, R., *Pflüger's Arch.*, 1900, **82**, 603.

[3] Smith, L., and Wolf, C. G. L., *J. Med. Research*, 1904, **12**, 451.

[4] Haurewitz, F., *Z. physiol. Chem.*, 1924, **68**, 138.

[5] Barnard, R. D., *J. Gen. Physiol.*, 1933, **16**, 657.

[6] Keilin, D., *Proc. Roy. Soc. London, B*, 1933, **113**, 393.

[7] Barnard, R. D., *J. Biol. Chem.*, 1937, **120**, 177.

[8] Pauling, L., and Hendricks, S. B., *J. Am. Chem. Soc.*, 1926, **48**, 1926.

[9] Barnard, R. D., and Neitzel, W., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 171.

[10] Barnard, R. D., and Neitzel, W., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 462.

[11] Jung, F., *Biochem. Z.*, 1940, **304**, 37.

[12] Hecht, G., *Biochem. Z.*, 1940, **305**, 290.

[13] Linhard, M., and Betz, K., *Ber.*, 1940, **73B**, 177.

[14] Barnard, R. D., *J. Lab. and Clin. Med.*, 1942, **27**, 774.

[15] Barnard, R. D., *J. Am. Pharm. Assn.*, in press.

[16] Pauling, L., and Coryell, C. D., *Proc. Nat. Acad. Science*, 1936, **22**, 159.

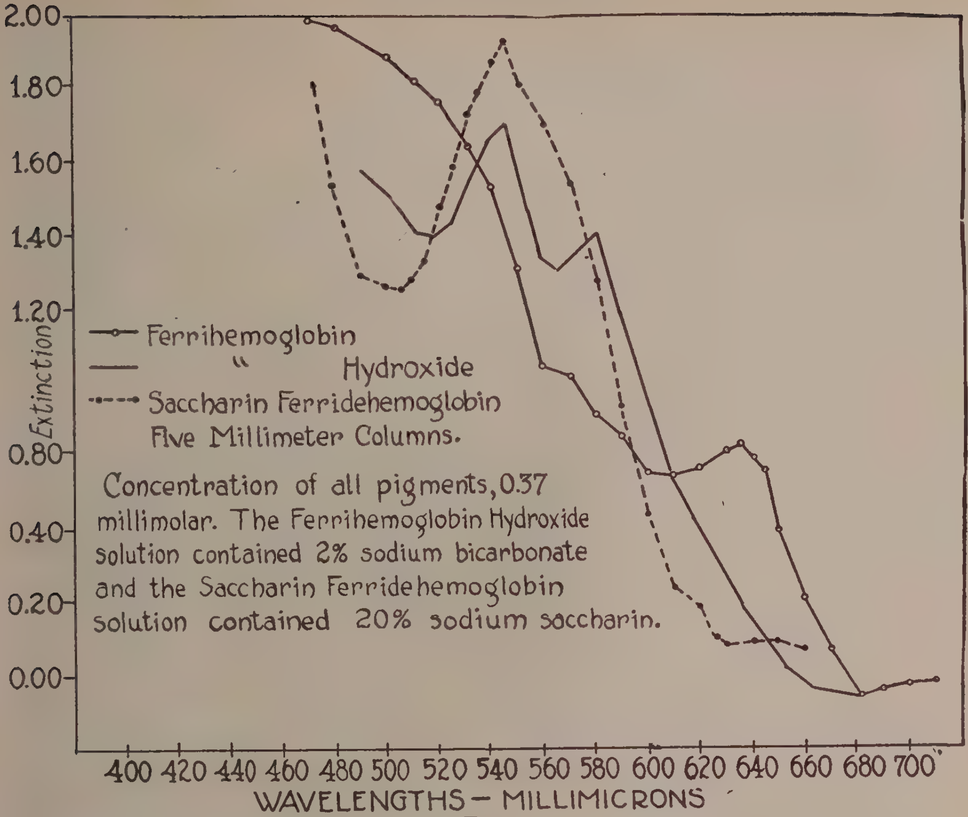

FIG. 1.
Spectrophotometric absorption curves of ferrihemoglobin, ferrihemoglobin hydroxide and saccharin ferridehemoglobin.

Whether or not this hypothesis is the correct one, its utility is beyond question for it has led to the prediction of the existence of several members of a class of blood pigment derivatives. An additional member, a compound of ferrihemoglobin with o-benzoic sulphinide (saccharin) is the subject of this report.

When soluble saccharin is added to a solution of ferrihemoglobin, the color of the latter changes to a bright red. This change is not complete until there is present in the solution a relative molar ratio of five hundred of saccharin and is not apparent until this ratio is over one hundred. If the ferrihemoglobin solution has been buffered at pH 6 so that all of the pigment is in the acid form, a visible color change is produced by 10 molar equivalents of saccharin but the latter also induces rapid denaturation and precipitation at this pH so that spectrophotometry on such acid specimens is out of the question. The curves shown were therefore obtained at pH 7.4.

The color of the solution of the saccharin-ferrihemoglobin reaction product bears a superficial resemblance to that of ferrihemoglobin hydroxide but the formation of the latter substance in the former solution was ruled out by the absence of any change in pH of the ferrihemoglobin solutions on the addition of soluble saccharin as well as by the distinction in the respective spectrophotometric curves. Any possible role which salt effect of the high concentrations of sodium saccharin employed might have exerted in the production of the color change, was ruled out by half saturation of control samples of ferrihemoglobin with dextrose, sodium chloride and ammonium sulfate, in which instances the molarity of such solutions was about twice that existing in the saccharin-ferrihemoglobin solution. No visible or spectroscopically detectable alteration was produced.

On the basis of the facts that soluble saccharin is the sodium salt of a hydro-acid, o-benzoic sulphinide; that it reacts with ferrihemoglobin in solution to produce a colored entity which is similar in its spectroscopic picture to that of the class of blood pigment derivatives termed ferridehemoglobins, the saccharin derivative is designated as saccharin- or o-benzoic sulphinide ferridehemoglobin.

Like certain other ferridehemoglobin formers (fluoride, fulminate, and cyanate) saccharin does not form ferridehemochromogens. The apparently high degree of dissociation of saccharin ferridehemoglobin which would seem to be indicated by the relatively high concentrations of saccharin required for the complete conversion of ferrihemoglobin is similar to that exhibited by the ferridehemoglobins of cyanamide, cyanate, and thiocyanate while it differs markedly from that observed in the cases of the ferridehemoglobins formed by the highly toxic anions, azide, cyanide, and fluoride. There appears to be some parallelism in the degree of association of hydro-acid anions with ferrihemoglobin and their inhibitory effect on the blood catalase. In this connection it is found that saturation with soluble saccharin is without appreciable effect on the catalase activity of whole blood, plasma or hemoglobin solutions. From the high concentrations of soluble saccharin found necessary for the completion of its reaction with the prosthetic nucleus of ferrihemoglobin, it is doubtful if this reaction is of any toxicologic importance.

14341

The Anti-Bacterial Action of the Xanthine Oxidase System.*†

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Notatin, an anti-bacterial substance isolated from *Penicillium notatum*, has been shown by Raistrick and coworkers[1] and others[2,3] to be a flavoprotein which catalyses the aerobic oxidation of glucose to gluconic acid with production of H_2O_2. According to these authors H_2O_2 is the actual anti-bacterial agent and the function of the flavoprotein-glucose-oxygen system is merely that of providing a continuous albeit minute supply of H_2O_2. It follows that any flavoprotein system which gives rise to H_2O_2 should have at least qualitatively the same anti-bacterial action as notatin. We have tested this hypothesis with the flavoprotein from milk which catalyses the oxidation of hypoxanthine to uric acid, and the results clearly indicate that there is little to choose between the flavoproteins from cow's milk and *Penicillium notatum* respectively as far as anti-bacterial action is concerned. The sole difference is that notatin is a somewhat more stable enzyme.

Experimental. Milk flavoprotein was prepared in purified form by the method of

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* This investigation was aided by grants from the Research Corporation, Nutrition Foundation, Rockefeller Foundation, and Lederle Laboratories.

† Since our article was submitted for publication on June 25, 1943, there has appeared in *Science* for September 10, 1943, an article by Lipmann and Owen who made observations comparable to those which we are reporting.

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[3] Kocholaty, W., *Science*, 1943, **97**, 186.

TABLE I.

Anti-bacterial Action of the Xanthine-oxidase System on the Growth of *Staphylococcus aureus*. Experiments Were Carried Out Aerobically at 38° in 1% Peptone Medium (Difco No. 13123). Final Concentration of Hypoxanthine Was 1 mg in 5 cc of Medium.

Additions to medium	Production of uric acid at 16 hr	Growth after hr				
		16	24	40	64	88
None	0	++++	++++	++++	++++	++++
Hypoxanthine	0	++++	++++	++++	++++	++++
Xanthine oxidase (0.03 unit)	0	++++	++++	++++	++++	++++
Hypoxanthine + xanthine oxidase (0.03 unit)	++++	0	0	0	0	0
Hypoxanthine + xanthine oxidase (0.003 unit)	+	0	0	0	0	0
Hypoxanthine + boiled xanthine oxidase (0.03 unit)	0	++++	++++	++++	++++	++++

TABLE II.

Concentration-activity Relations for Anti-bacterial Action of the Xanthine Oxidase System on Aerobic Growth of *Staphylococcus aureus* in Casein Hydrolysate-Beef Broth at 38°.

Final conc. of enzyme (parts pure enzyme to parts of water)	16 hr	20 hr	24 hr	40 hr	64 hr
0	++++	++++	++++	++++	++++
$1:10^4$	0	0	0	0	0
$1:10^5$	0	0	0	0	0
$1:10^6$	0	0	0	0	+
$1:10^7$	0	0	0	+	++++
$5:10^8$	0	0	+	++++	++++
$1:10^8$	0	+	++	++++	++++
$1:10^8$ (renewed every 12 hr)	0	0	0	0	0

Corran *et al.*[4] One Corran unit of xanthine oxidase activity is equivalent to 600 γ flavoprotein containing 1.6 γ flavinphosphate. Thus by estimating the enzymic activity of our purified, sterile solutions in terms of Corran units we were able to determine the precise concentration of active flavoprotein. It is important to note that sterilization by filtration through a Seitz filter led to great losses of activity owing to contamination by heavy metal. Chamberland candles proved more satisfactory.

The substrate for the milk enzyme (xanthine or hypoxanthine) is present in abundance in the meat broth media commonly used in bacteriological studies. Further addition of substrate is therefore superfluous. Peptone water or hydrolysates of purified proteins, however, contain neither hypoxanthine nor xanthine and were used for the experiments demonstrating that the anti-bacterial action of the xanthine oxidase is dependent upon the presence of its substrate.

Oxygen must be present in the medium for the enzymic oxidation to take place. We have found in practice that an adequate oxygen tension obtains in the growth medium when the layer of fluid does not exceed 2 cm in depth.

Table I shows that the aerobic growth of *Staphylococcus aureus* in peptone water is inhibited by milk flavoprotein only when hypoxanthine is added. The boiled enzyme in presence of substrate is inactive. That the anti-bacterial action is due to the functioning of the xanthine oxidase is shown by the fact that uric acid is formed in appreciable quantity only when the complete enzyme system is present. This coincides with the suppression of bacterial growth. Under conditions of anaerobic growth which do not permit the xanthine oxidase to function, no anti-bacterial action is observed. The anti-bacterial action of milk flavoprotein on the growth of *Staphylococcus aureus* is demonstrable at a final dilution of 10^8 over a 16-hour period (Table II). The enzyme is unstable under the experimental conditions, and at the end of a

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[4] Corran, H. S., Dewan, J. G., Gordon, A. H., and Green, D. E., *Biochem. J.*, 1939, **33**, 1694.

TABLE III.
Reversal by Catalase of Anti-bacterial Action of the Xanthine Oxidase System on Growth of *Staphylococcus aureus.* The experiments were carried out aerobically at 38° in casein hydrolysate-beef broth.

Addition to medium	Growth after 16 hr	Growth after 40 hr	Growth after 64 hr
None	++++	++++	++++
Xanthine oxidase (0.05 unit)	0	0	0
” ” + catalase sol. diluted 1:25	++++	++++	++++
” ” + ” ” ” 1:250	++++	++++	++++
” ” + ” ” ” 1:2500	+	++++	++++
” ” + ” ” ” 1:25,000	0	++++	++++
” ” + ” ” ” 1:250,000	0	0	0

TABLE IV.
Reversal by Azide and Hydroxylamine of Catalase Inhibition of Anti-bacterial Action of Xanthine Oxidase (X.O.) on Growth of *Staphylococcus aureus.*

Additions to medium	Growth after 40 hr	Growth after 80 hr
Xanthine oxidase (0.05 unit)	0	0
X.O. + catalase (diluted 1:2500)	++++	++++
X.O. + ” + M/100 hydroxylamine	0	0
X.O. + ” + M/200 ”	0	0
M/100 hydroxylamine	+	++++
M/200 ”	++	++++
X.O. + catalase + M/1000 azide	0	0
X.O. + ” + M/2000 ”	0	0
M/2500 azide	++	++++
M/5000 ”	++++	++++

few hours only a small fraction of the original activity survives. The effective concentration of enzyme during the experimental period is therefore much less than the initial.

The anti-bacterial effect of milk flavoprotein at the lower limits of concentration is one of bacteriostasis. Growth is suppressed so long as the enzyme system produces H_2O_2. As soon as the activity of enzyme ceases for whatever reason, growth of the bacteria proceeds normally. Thus a concentration of enzyme at the lower limit of effectiveness will stop growth for 16 hours. If the concentration of enzyme is renewed every 12 hours growth can be suppressed for days. However, growth starts promptly as soon as one renewal is missed out.

That H_2O_2 is indeed responsible for the bacteriostasis is shown by the fact that minute concentrations of catalase, an enzyme which decomposes H_2O_2, reverse the anti-bacterial action of the xanthine oxidase (Table III). In turn by adding azide or hydroxylamine as catalase inhibitors the anti-bacterial action is restored. The conditions for obtaining this azide and hydroxylamine inhibition of the catalase reversal are critical and are given in Table IV. We have never been able to demonstrate the anti-bacterial action of milk flavoprotein on the growth of *Staphylococcus aureus* in serum but this failure has been correlated with the presence of high concentrations of catalase in serum.

Milk flavoprotein inhibited the growth of *M. catarrhalis, Bact. shiga, Proteus vulgaris, Pseudomonas pyocyanea, M. sarcina lutea, Streptococcus haemolyticus* (5 strains), and *B. subtilis* in addition to some 10 strains of *Staphylococcus aureus* and *albus.* It had no action even at high concentrations on the growth of *Bact. coli, Bact. friedlanderi, Bact. lactis aerogenes, Bact. paratyphosus* B, *Streptococcus pneumonia* A 66, *Bact. flexneri* and *Corynebacterium diphtheriæ.*

Summary. The anti-bacterial action of the milk xanthine oxidase system has been shown to be due to formation of H_2O_2.

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Blood Studies on Dogs Subjected to Discontinuous Exposure to Low Oxygen Tension.

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Much of our knowledge of the effects of anoxic anoxia has been gained through studies of the experimental subject exposed continuously to low oxygen tensions, while relatively little has been obtained of the changes during discontinuous exposure. Since the latter condition prevails in aviation, at least up to the altitude above which oxygen is administered, and since it obviously presents different problems to the organism it is a worthwhile subject to study. Accordingly we have expanded our investigations of the changes occurring in the blood under this condition.

Methods. Five male dogs weighing 7.83 to 11.92 kg were the experimental animals. They were maintained on an adequate diet throughout the period of study, and the amount fed daily was adjusted to maintain their body weight practically constant. It has been our experience that during the period of exposure to anoxia the amount fed must be considerably increased to maintain the dogs in the same state of nutrition.

After a preliminary control period the dogs were exposed 8 hours daily (except Sundays) by means of a low pressure chamber to a partial pressure of oxygen of 80 mm Hg (18,000 ft) for a period of 88 days. Blood studies were made at weekly intervals. Blood specific gravity was determined on freshly drawn samples of venous blood by the falling drop method of Barbour and Hamilton.[1] Hemoglobin was determined by the Sahli method, and red blood cells were counted in the improved Neubauer counting chamber. At monthly intervals plasma protein was determined on 3 of the dogs by a modification of the method of Guillaumin, Wohl, and Laurencin.[2] The control and the final values for plasma protein for each dog were determined on 4 separate blood samples drawn over the course of a week, while those for the first and second months were determined on 2 separate samples. Red blood cell fragility tests were made in duplicate at the conclusion of the experimental period.

Results and Discussion. The fragility of the red blood cells was normal; hemolysis began at 0.46 and was complete at 0.33% NaCl. This finding is at variance with those of Hurtado[3] on native Peruvian Indians who live at high altitudes, and of Minot and Buckman[4] in polycythemia vera. An increased resistance to hypotonic NaCl was found by these workers.

In Fig. 1 the mean percent changes in the number of red blood cells, hemoglobin values and plasma protein are plotted against time from the beginning of the period of discontinuous anoxia. The mean specific gravity values are plotted directly, with the control value set at the zero point of the ordinates for percent change, and the scale for specific gravity arbitrarily chosen to make the curve fall approximately with the hemoglobin curve.

The production of hemoglobin and the increase in number of red blood cells were practically parallel until the end of the fourth week of exposure to discontinuous anoxia. From them on hemoglobin production failed to keep pace with the red blood cell increase, and at the end of the period of anoxia, the hemoglobin value had increased 53% while red blood cells had increased 67%. However, the curves (not reproduced here) for the individual dogs show this characteristic in a definite way in but 2 out of the 5 cases. In

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[1] Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

[2] Guillamin, C. O., Wohl, R., and Laurencin, M. L., *Bull. soc. chim. biol.*, 1929, **11**, 387.

[3] Hurtado, A., *Am. J. Physiol.*, 1932, **100**, 487.

[4] Minot, F. R., and Buckman, T. E., *Am. J. Med. Sci.*, 1923, **166**, 469.

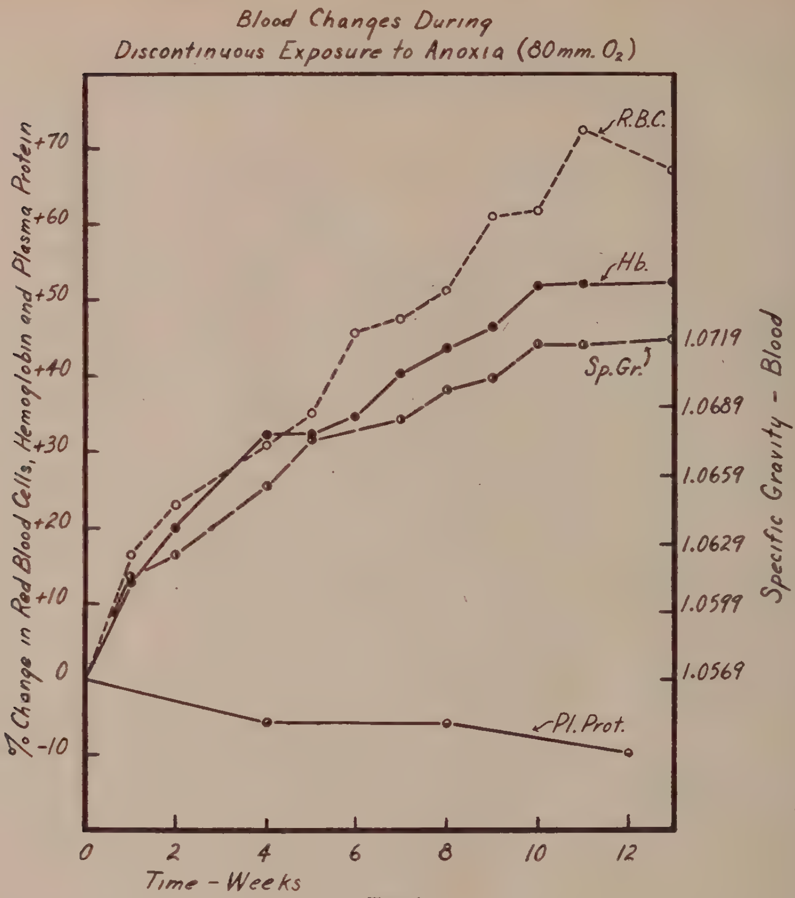

FIG. 1.

3 of the dogs a rough parallelism extended throughout the experimental period.

The concentration of plasma protein decreased slightly over the experimental period and at the end of the third month had decreased to approximately 10% below the control level. Statistical analysis of the data proved this to be insignificant for the group of 3 dogs. However, in the case of one dog, analyses of the series of determinations done each month showed a significant decrease throughout.

The average value for specific gravity increased from 1.0569 to 1.0719 during the period of discontinuous anoxia. A comparison of the curve for specific gravity with that for hemoglobin suggests that the former fails to reflect all the changes in the latter. It seems probable that the decrease in plasma protein (although but 3 of the 5 dogs are represented) would account for much of this divergence.

Summary. Five dogs exposed 8 hrs daily to an O_2 tension of 80 mm Hg (18,000 ft) for 88 days showed no change in red blood cell fragility. Hemoglobin values increased 53%, while the number of red blood cells increased 67%. The specific gravity of the blood rose from 1.0569 to 1.0719. Plasma protein concentration decreased 9.69%.

14343

Vitamin B_1 Deficiency and Attempts to Produce Poliomyelitis in White Rats.

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The experiments reported in this paper are the first of a series, the purpose of which is to determine whether Flexner's M.V. strain, which has been acclimated to Eastern cotton rats, could in turn be acclimated to white rats by some vitamin deficiency or imbalance.

This first series was with B_1-deficient animals. White rats were fed a vitamin B_1 test diet made up as follows: sucrose—62.1%; vitamin free casein—18.6%; peanut oil—7.8%; salt mixture—4.2%; cod liver oil—2.3%; and alkaline autoclaved brewer's yeast—5.0%. When the animals first started this diet, they were from 22 to 26 days old and weighed between 40 and 50 g.

We had ascertained that white rats from our colony had to be kept on this diet 30 days before they developed full-blown B_1 deficiency. If the diet were continued the animals would start to die beginning about the thirty-eighth day. This left 8 days in which to demonstrate additive effects of poliomyelitis virus. Signs and symptoms should appear after the fifth or at least before the eighth day following injection of virus, which occurred on the thirtieth day of the diet. There was sufficient time since reactions begin to appear in Eastern cotton rats about the fourth to fifth day after injections with Flexner's M.V. cotton rat-adapted strain.

A second difficulty arose. Vitamin B_1-deficient rats have a polyneuritis. It might be difficult to distinguish this condition from that produced by poliomyelitis virus, especially since the early clinical appearance of white rats which are vitamin B_1-deficient and cotton rats infected with poliomyelitis are alike, *i.e.,* they are furred, have tremors, apraxia and weakness. There are two definite points of differentiation. Twisting the tail of vitamin B_1-deficient animals sends them into a typical rolling movement not seen in poliomyelitis virus-infected animals that have paralysis; vitamin B_1-deficient rats never go on to a stage of typical flaccid paralysis with dragging of the hind legs as occurs in cotton rats after virus injections.

Experiment 1 (a). Before injecting virus suspension, a number of materials, such as normal cotton rat brain-cord suspensions, normal white rat brain-cord suspensions, etc., had to be investigated for their non-specific reactions.

The dose of specific or non-specific suspension always given in this and the following experiments was: 0.06 cc intrathecally, 0.06 cc intranasally, 0.5 cc subcutaneously, and 1.0 cc intraperitoneally. Brains and cords were always ground in normal saline for the test dose.

To conserve space, vitamin B_1-deficient white rats will be termed "deficient" rats and Flexner's M.V. cotton rat-adapted strain suspension will simply be termed "virus suspension." Normal cotton rat brain cord suspension will be termed "normal suspension." Brain and cord suspension from deficient rats will be termed "deficient suspension."

"Normal suspensions" from white rats (1a, Table I), "normal suspensions" from cotton rats (1b, Table I), and enteric toxin sometimes used in our laboratory to enhance the virulence of virus (1h, Table I) were tested.

A latent virus might have been present in the brains and cords of "deficient" animals that died and sub-transfers were made of injected and uninjected "deficient suspensions" (1c and d, Table I) into other "deficient" animals.

Something might be catalyzed in the central nervous systems of rats fed deficient diets and an autocatalytic enzyme produced which would

TABLE I.
Reactions in White Rats to Injection with Control Materials.

				Results	
Exp. No.	Control injections	Into	Deficient white rats	Dead	Between days
1a	“Normal Suspension”	white rat	4	4	5-23
1b	” ”	cotton ”	10	10	9-23
1c	“Deficient Suspension”	white rats from Exp. 1b	4	4	8-27
1d	” ”	” ” ” ” 1c	10*	5	6-62
1e	” ”	5 killed white rats from Exp. 1d	5	5	19-25
1f	” ”	white rats from Exp. 1e	10	10	9-23
1g	” ”	” ” ” ” 1f	4	4	13-27
1h	Enteric Toxin		10	10	6-70

* 5 killed on the 10th day for transfer material.

TABLE II.
Attempt to Acclimate Flexner's M.V. Rat Adapted Strain to White Rats.

				Results	
Exp. No.	Virus Injections	Into	Deficient white rats	Dead	Between days
2a	“Virus Suspension”		4	4	15-30
2b	“Deficient Suspension”	white rats virus inj., Exp. 2a	11	10*	6-16
2c	” ”	” ” Exp. 2b	5	5	12-19
2d	” ”	” ” ” 2c	10	9†	8-23
2e	” ”	” ” ” 2d, showing weakness	6	6	2-7
2f	” ”	” ” ” 2e	5	5	5-28
2g	” ”	” ” ” 2f	10	10	6-13
2h	” ”	” ” ” 2g	10	10	8-41

*1 died first day from accident.
†1 died thirteenth day—had weakness.

in turn cause paralysis in other “deficient” rats. Serial transfers were therefore made from “deficient” rats into other “deficient” animals (1e, 1f, and 1g, Table I.)

Results. The results are outlined in Table I. It could be concluded that no factor, either latent or otherwise, was found in the central nervous system of either normal or vitamin B_1-deficient white rats or normal cotton rats which would cause paralysis in white rats; nor did enteric toxin cause any such reaction.

In the next experiment “deficient” animals were injected with “virus suspension” and when these animals died an emulsion of their cords in saline was injected into “deficient” white rats. Serial transfers were attempted as outlined in Table II.

One of the animals of Experiment 2d that died on the thirteenth day had questionable weakness of the hind legs. Cord suspensions of this animal injected into other “deficient” animals (2e, Table II), did not produce paralysis, although these animals died sooner than did those in previous experiments. This was suggestive since it resembled the way in which other virus strains affected the Eastern cotton rat as they become acclimated to that animal. If this represented a beginning acclimation, the next series of injections would probably produce paralysis at least in some animals. Three more generations were tried without producing any such result.

Results. In the virus injection experiment, there was suggestive weakness in only one animal which may have been due to injury. Under the conditions of our experiment, there was no evidence that vitamin B_1 deficiency would in any way make white rats more susceptible to Flexner's M.V. poliomyelitis cotton rat-adapted strain.

14344

Quantitative Relation Between Administered Alkali and Endogenous Citric Acid Production.

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Östberg[1] proposed an hypothesis that, in human metabolism, the citrates of the urine constitute one of the important buffer systems. This suggestion evoked considerable investigation relative to the possible function of citric acid as a "metabolic acid" in much the same sense that ammonia is a "metabolic base." This theory has been supported by the establishment of the endogenous origin of citric acid, as shown by its synthesis in the animal organism from various precursors.[2,3] Furthermore, when citric acid is administered as the free acid to human subjects[4-8] and to experimental animals[9-14] it disappears as such, whereas the ingestion of sodium citrate leads to the appearance of relatively large amounts of citric acid in the urine.[1,7,9] That this difference is due principally to the alkali administered is indicated by the fact that equivalent amounts of sodium, as the bicarbonate, produce similar increases in citric acid excretion.[1,4-7,9] Conversely, the administration of hydrochloric acid and potentially acidic substances, such as ammonium chloride, cause a significant decrease in the excretion of the acid.[1,4,5] It is evident from these investigations that the endogenous production of citric acid is highly responsive to factors known to affect the recognized metabolic adjustments to changes in acid base equilibrium in the body.

In view of the evidence relative to the qualitative stimulation of citric acid production by alkali, research was inaugurated in an attempt to establish a possible direct relationship between the excretion of citric acid and the amount of alkali administered. Seven male albino rats, weighing from 200-250 g, were maintained over the experimental period on either of 2 low-citrate, iso-caloric diets. Diet Cbh contained Lactalbumin (Labco, No. 15-42, Borden) 14.0%, Dextrin 58.6%, Hydrogenated Fat (Crisco) 20%, Cod Liver Oil (Mead, Johnson & Company) 5%, Salt Mixture[15] 2.4%, while diet Pr consisted of Lactalbumin 72.6% and no dextrin—the remainder of the purified ration being the same as diet Cbh. The caloric values were 5.24 cal. per g for the Cbh diet and 5.32 cal. per g for the Pr ration. The daily supplements were 1 cc of a solution containing 80 mg of liver extract (Lilly 343) and 200 mg of Ryzamin-B concentrate (Burroughs Wellcome).

The animals were kept in individual cages placed over large glass funnels containing a 3/16-inch mesh screen and glass wool plugs which separated the feces and food from the

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TABLE I.
Alkali Administration and Endogenous Citric Acid.

		Mg citric acid per period			Urinary citric acid excretion			
Diet	Periods on diet	Intake* A	Urinary excretion B	Mg $NaHCO_3$ per period C	Excr. per 100 mg $NaHCO_3$ D	Total incr. over norm E	Incr. per 100 mg $NaHCO_3$ F	Avg incr. per 100 mg $NaHCO_3$ G
Cbh R	5	6.06	8.49	—	—	—	—	—
" R B_1	3	5.99	14.49	150	9.66	6.00	4.00	4.73
" R B_2	3	5.98	23.33	300	7.78	14.84	4.95	
" R B_3	3	6.23	39.90	600	6.65	31.41	5.24	
Pr R	3	26.66	11.96	—	—	—	—	—
" R B_1	3	27.82	23.39	150	15.60	11.43	7.66	6.16
" R B_2	2	27.82	28.83	300	9.61	16.87	5.62	
" R B_3	3	27.82	43.21	600	7.20	31.25	5.21	
							Mean	5.45

* Analysis for Exogenous Citric Acid:
Cbh—0.166 mg per g.
Pr—0.916 mg per g.
Suppl. vitamin—0.51 mg.

urine, which was collected in a flask containing approximately 25 cc 1 N H_2SO_4. The funnels were rinsed down daily with distilled water and the combined urine and washings were diluted to 250 cc. Appropriate aliquots were analyzed for citric acid by the method of Pucher, Sherman, and Vickery,[16] the final colorimetric measurement being made in a photelometer using a filter with maximum transmission at 4250 Å. Quantitative measurements of food consumption, as well as of urinary citric acid, were made at period-intervals of 3 days.

After preliminary "adjustment" periods, to establish the caloric requirement for the individual animal, the rats were placed on a restricted diet "Cbh R and Pr R", so that consumption of food was complete or nearly so. In this series of experiments, the animals were used as "internal controls"—that is, each rat served as its own control. A "norm" was established first on the Cbh R diet after an adjustment period of 10 days. A solution of sodium bicarbonate was then administered orally by incorporation with the vitamin mixture to insure complete consumption. A similar "norm" was established for the Pr R diet and the effect of alkali on endogenous citric acid studied in an analogous manner. During all periods food intakes were iso-caloric for the individual animal. The results of experiment are found in Table I, each of the figures represents a period (3-day) average for the seven animals on the specified diet over the number of periods indicated.

In Table I, Column A represents the total citric acid intake per rat per period, while Column B shows the urinary excretion. In all cases dietary citric acid is relatively constant on the diet employed, while the increase of total urinary citric acid is dependent upon the degree of alkalosis. Calculation of the urinary citric acid excretion per 100 mg of $NaHCO_3$, Column D, illustrates a decrease in the effect of alkali as the amount of bicarbonate is increased. When the urinary citrate is calculated as the increment over the "norm," Column E, the linear relationship between administered alkali and urinary citrate becomes evident. The "norm" value for the Pr diet is higher than expected, due probably to a "carry over" from the previous experiments, even over an intervening adjustment period of 3 weeks. Since, however, the animals served as their own controls, this effect would not alter subsequent results to any major degree. Development of the possible direct relationship between the citric acid and $NaHCO_3$ is particularly evident in Columns F and G, where the average citric acid increments on both the Cbh and Pr diets are of the same order of magnitude.

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[16] Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, 1936, **113**, 235.

The foregoing data indicate that there is a direct relation between the amount of alkali administered to the rat and the excretion of endogenous citric acid. It would appear therefore that under the present experimental conditions, citric acid plays an important role in the regulatory mechanism involved in the acid-base balance of the body.

14345

Metabolic Studies in Patients with Cancer of Gastro-Intestinal Tract. XV. Lipotropic Properties of Inositol.[*]

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Recent studies from this hospital have demonstrated that the livers of patients with gastro-intestinal cancer almost uniformly are infiltrated with fat.[1] In contrast to this finding, normal fat contents were found in the livers of 11 patients with carcinoma of the gastro-intestinal tract who each received 8 g of lipocaic[†] during the night prior to laparotomy. The average hepatic fat concentration of this latter group was only 0.46 that of the control untreated patients.[2]

Lipocaic, however, is a crude mixture which contains significantly large amounts of choline and inositol,[†] both of which are lipotropic alone.[3,4] In experimental animals, the lipotropic effects of lipocaic apparently are not due to its choline alone,[5] and this observation has been confirmed here for human subjects.[2] On the other hand, when about 4 times the amount of inositol present in the 8 g of lipocaic was administered to a group of 8 patients with gastro-intestinal cancer, with but one exception the concentrations of fat in their livers were found to be within normal limits. The average lipid value was 0.42 that of the control group.

TABLE I.
Concentration of Fat in Livers of Patients with Gastro-intestinal Cancer Who Received Preoperatively 280 mg of Inositol.

Patient	Disorder	Conc. of hepatic fat, g per 100 g wet tissue
J.K.	Carcinoma oesophagus	7.2
R.H.	” stomach	8.7
O.S.	” colon	7.1
M.S.	” stomach	10.3
M.T.	” colon	7.8
W.R.	” stomach	5.7
H.J.	” ”	5.0
F.M.	” ”	15.9
G.M.	” ”	5.9
M.J.	” ”	8.9
	Avg	8.25

Because it seemed probable then that the inositol alone might account for the physio-

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[*] The authors gratefully acknowledge the support of the National Cancer Institute.

[†] The lipocaic used in this investigation was supplied through the courtesy of Eli Lilly & Co., Indianapolis, Ind., and the inositol through the kindness of Lederle & Co., Pearl River, N.Y. The concentration of choline and of inositol in the material used was found by analyses to be 2.6 and 3.5 g % respectively. These analyses were made by Dr. W. Wooley of the Rockefeller Institute for Medical Research, New York City, and by the Laboratories of the Standard Brands, Inc., New York City.

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TABLE II.
Average Concentration of Fat in the Livers of Patients with Gastro-intestinal Carcinoma Who Were Fasted and of Those Given Various Dietary Supplements Preoperatively.

No. of patients	Preoperative supplement	Avg conc of hepatic fat, g per 100 g wet tissue	% reduction of hepatic lipid effected by supplement
28	None	16.4	—
11	8 g lipocaic	8.0	51
7	3 g choline chloride	11.2	39
8	1200 mg inositol	6.9	58
10	280 ” ”	8.2	50

logic effects of the lipocaic, another group of 10 patients with carcinoma of the gastro-intestinal tract was given during the last 10 preoperative hours only that amount of inositol (280 mg) contained in the effective dose of lipocaic. The clinical and chemical methods used have been described in a preceding communication of this series.[1] The results obtained from the ingestion of the smaller amounts of inositol indicate that in the patients studied, the lipotropic properties of the lipocaic could be accounted for by its content of inositol alone (Tables I and II). Of the values of hepatic lipid, only one was significantly elevated, and the average of the group was only 0.50 that of the control group.

14346

Titration of St. Louis Encephalitis Virus and Jungeblut-Sanders Mouse Virus in Tissue Culture.[*]

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Following successful titration and neutralization of the Western strain of equine encephalomyelitis virus in chick embryo tissue culture,[1,2] attempts were made to see whether or not the same method could be applied to other viruses. The St. Louis encephalitis virus and the Jungeblut-Sanders mouse virus[3] were chosen for study.

The principle on which titration of the equine virus in tissue culture is based, is the finding that tissue fails to grow when it is heavily infected with virus. Thus, in the earlier studies when St. Louis encephalitis virus and Jungeblut-Sanders mouse virus were tested in chick embryo tissue cultures, it was found that potency could not be determined as simply as with the equine virus. There was no appreciable difference in the growth of infected and non-infected tissue when the tissue was subsequently patched with plasma.

Since it was felt that the failure to note a difference in growth of infected and non-infected tissue treated with St. Louis encephalitis and Jungeblut-Sanders mouse viruses was probably due to low susceptibility of chick embryo tissue to these 2 viruses,[2] it became of interest to determine whether or not a difference in the growth of infected and non-

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[†] The author wishes to express his appreciation to Major M. Sanders, Dr. A. R. Dochez, and Dr. W. W. Palmer for their constant encouragement and interest.

[1] Huang, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 396.

[2] Huang, C. H., *J. Exp. Med.*, 1943, **78**, 111.

[3] Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

TABLE I.
Comparison of *in vitro* and *in vivo* Titration of St. Louis Encephalitis Virus and Jungeblut-Sanders Mouse Virus.

Virus used	Titrated in	Virus dilution tested								
		No virus	10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9
St. Louis encephalitis virus	Mice	A A	4 4	4 4	5 5	5 6	A A	A A	A A	
		A A	4 5	4 4	5 5	6 A	A A	A A	A A	
	Tissue culture	OOOO	GGGG	GGGG	GGGG	GGGG	OOOO	OOOO	OOOO	
		OOOO	GGGG	GGGG	GGGG	GGGG	OOOO	OOOO	OOOO	
Jungeblut-Sanders mouse virus	Mice	A A	2 2	2 2	3 3	3 3	3 3	3 4	A A	A A
		A A	2 2	3 3	3 3	3 3	3 4	4 4	A A	A A
	Tissue culture	OOOO	GGGG	GGGG	GGGG	GGGG	GGGG	GGGG	OOOO	OOOO
		OOOO	GGGG	GGGG	GGGG	GGGG	GGGG	GGGG	OOOO	OOOO

A = Mouse remained alive 8 days.

Each number indicates day of death of the animal after the infection and presumably represents a virus death.

G = Growth of fibroblasts from explants noted.

O = No growth from the explants.

Each "G" or "O" represents growth activity of a single explant. Eight explants were used for each plasma preparation.

infected tissue could be demonstrated if a more susceptible tissue were employed. Embryonic mouse brain tissue was therefore chosen since adult mice succumbed regularly to intracerebral infection with these 2 viruses in high dilution. As the growth of brain tissue in plasma preparation has not been satisfactory, the following method has been introduced.

Mouse brain tissue containing the virus (either St. Louis encephalitis virus or Jungeblut-Sanders mouse virus) was diluted serially in physiological solution "Z_2."[4] One drop of the material from each dilution was inoculated into tissue cultures consisting of a series of tubes each containing 10 pieces of minced embryonic mouse brain tissue (the embryo measured 8-10 mm in length) and 1 cc of diluted serum ultrafiltrate.[4] The entire preparation was incubated at 37.5°C for 4 days in the case of the Jungeblut-Sanders mouse virus and 6 days when St. Louis encephalitis virus was tested. After incubation, one drop of 10 M.L.D. of Western strain of equine encephalomyelitis virus was introduced into each tube which was then re-incubated. At the end of 48 hours, one drop of the supernatant fluid from each tube was inoculated into separate tubes each containing 10 pieces of minced skeletal muscle of 9-day developing chick embryo and 1 cc of serum ultrafiltrate diluted in a physiological solution. They were then kept at 37.5°C. After 48 hours of incubation, pieces of tissue from each tube were transferred and patched with plasma in separate Carrel dishes. Readings were made under the low power microscope after 24 hours of incubation.

It was thus found that embryonic mouse brain tissue infected with either the St. Louis encephalitis virus or the Jungeblut-Sanders mouse virus incubated for 6 and 4 days respectively, failed to support the growth of 10 M.L.D. of Western strain of equine encephalomyelitis virus. This is shown by the fact that the equine virus disappeared from these cultures after 48 hours of incubation. Consequently, when the supernatant fluid from these cultures was subsequently inoculated into chick embryo tissue cultures, the chick embryo tissue remained uninfected by equine encephalomyelitis virus and was able to proliferate when the tissue was patched with plasma. Since it has been found that both the St. Louis and the Jungeblut-Sanders mouse viruses have no influence on the growth of chick embryo tissue and therefore they do not interfere with the test, no attempt was made to see whether or not these viruses were still present in these cultures after the introduction of equine virus. Conversely, when the embryonic mouse brain tissue was not infected by either test virus, the tissue promoted the growth of the equine virus in such a way that when the supernatant fluid of these cultures, after 48 hours of incubation, was inoculated into chick embryo tissue cultures, the tissue became infected and failed to grow when it was plasma-patched.

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[4] Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **33**, 619.

The sensitivity of the *in vitro* titration method presented here was compared with intracerebral mouse inoculation. Soon after the serially diluted virus was inoculated into tissue culture, 0.03 cc of the material from each dilution was inoculated intracerebrally into mice. The results are shown in Table 1. It can be seen that the titer obtained in the tissue culture is the same as that of the intracerebral mouse inoculation test. The test was repeated 3 times with approximately similar results.

The method herein presented is rather complicated and can hardly be applied for mass study. However, it suggests the possibility of detecting virus in tissue culture if a highly susceptible tissue is employed. Another method which obviates this complex procedure has been developed and will be reported separately.[5]

Conclusion. Both the St. Louis encephalitis virus and the Jungeblut-Sanders mouse virus have been successfully titrated *in vitro* by the use of embryonic mouse brain tissue and with the help of another system consisting of chick embryo tissue and Western strain of equine encephalomyelitis virus.

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[5] Huang, C. H., PROC. SOC. EXP. BIOL. AND MED., in press.

14347 P

A Visible Method for Titration and Neutralization of Viruses on the Basis of pH Changes in Tissue Cultures.*

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The advantages of determining virus potency and of demonstrating neutralizing antibodies of the Western strain of equine encephalomyelitis (W.E.E.) virus in tissue culture as presented in previous papers[1,2] include (a) reduction of cost of experiments; (b) removal of the variable of individual animal reactivity; and (c) the possibility that the tissue culture method is a more sensitive indicator of the presence of virus than animal inoculation. However, the application of the method to other viruses was limited, since the titration and neutralization of the W.E.E. virus depended upon growth, or absence of growth of tissue. Such a test could not be applied to many viruses because of the low susceptibility of the fibroblasts to virus. Thus, the St. Louis encephalitis virus and the Jungeblut-Sanders mouse virus[3] could not be titrated in chick embryo tissue culture and a more complicated procedure had to be developed when embryonic mouse brain, the most susceptible tissue, was used.[4] This complicated procedure was introduced because of the difficulty of growing the mouse brain tissue

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† The author wishes to express his appreciation to Major M. Sanders, Dr. A. R. Dochez, and Dr. W. W. Palmer for their constant encouragement and interest.

[1] Huang, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 396.

[2] Huang, C. H., *J. Exp. Med.*, 1943, **78**, 111.

[3] Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

[4] Huang, C. H., PROC. SOC. EXP. BIOL. AND MED., in press.

in plasma patched preparations. To obviate the complex procedure, a simplified method has been developed.

During an investigation concerned with metabolism of infected and non-infected tissue, it was found[5] that the degree of glycolysis in the tissue infected with the W.E.E. virus as measured in the respirometer of a type described by Victor,[6] was much lower than that of non-infected tissue. On the basis of this observation, the present method for titration and neutralization of several viruses has been developed. The method does not require transfer of tissue into Carrel dishes and the whole experiment can be carried out in the same tube. The method is based on the fact that the products of glycolysis are acid in reaction. In preliminary experiments, it was found that when non-infected tissue was present, the pH of the culture medium shifted to the acid side and in the presence of phenol red, the color of the medium changed from red to yellow within 2 hours at 37.5°C in partial vacuum (a desiccating jar evacuated for one hour by water suction). Conversely, when tissue was heavily infected with virus for a sufficiently long period of time, there was either very little change or no change in the pH of the medium.

In order to obtain the greatest change in the pH of the medium, it was necessary to substitute a Ringer's solution containing 0.1% glucose and 5 mg % phenol red for the serum ultrafiltrate medium after the tissue cultures containing virus had been incubated for several days. The change of the medium was essential since diluted serum ultrafiltrate is a well-buffered medium and does not reflect pH changes as readily as the Ringer's solution. In this way, the potency of and neutralizing antibodies against the W.E.E. virus in chick embryo tissue culture could be calculated by making use of visible changes in the fluid medium. In the same manner, the St. Louis encephalitis virus and the Jungeblut-Sanders mouse virus could be titrated in embryonic mouse brain tissue culture in a relatively short period of time. There is evidence that the method is practicable and is accurate when compared with animal inoculation.

Method. Ten-fold dilutions of virus were made in a physiological solution "Z_2."[7] 0.1 cc of the material from each dilution was inoculated into tissue cultures consisting of a series of tubes each containing 15 to 20 pieces of minced embryonic tissue (mouse embryonic brain tissue for Jungeblut-Sanders mouse virus and St. Louis encephalitis virus; chick embryonic skeletal tissue for equine virus) and 1.5 cc of diluted serum ultrafiltrate.[7] The different mixtures were incubated at 37.5°C for different periods of time depending on the particular virus used (2-3 days if Jungeblut-Sanders mouse virus and equine virus were tested and 6-7 days when St. Louis encephalitis virus was used). After incubation, supernatant fluid of each tube was discarded and the tissue was washed twice with 5 cc of modified Ringer's solution. The washed tissue was finally suspended in approximately 0.3 cc of the modified Ringer's solution. The entire preparation was then kept in a desiccating jar and the air inside the jar was evacuated by water suction pump for 1 hour. After this period of evacuation, the desiccating jar containing the cultures was kept at 37.5°C and the readings were made in 2 to 4 hours.

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[5] Victor, J., and Huang, C. H., unpublished data.

[6] Victor, J., *Am. J. Physiol.*, 1935, **111**, 477.

[7] Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **33**, 619.

14348

Endosteal Bone Deposition in Femurs of Vitamin D-Deficient Mice Treated with Estrogen.

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It has been reported by a number of investigators[1] that the treatment of mice with estrogens produces an extensive proliferation of endosteal bone in the femurs which may result in the complete obliteration of the marrow cavity of these bones. This phenomenon is usually accompanied by resorption of the pubic bones and formation of a pubic ligament. As is well known, a similar resorption of the pubic bones occurs as a normal process during the reproductive cycle in the pocket gopher.[2]

It was of interest to determine whether the changes in the bones observed by others in mice treated with estrogens and maintained on adequate diets could be reproduced by similar treatment of mice fed food deficient in vitamin D. It seemed possible that vitamin D might play a role in the mechanism of deposition of osteoid tissue in the femur. It was, therefore, decided to study the effect of treatment with estrogenic hormone on mice fed vitamin D-deficient diets. The absence of changes in the femurs of mice treated in this manner would implicate vitamin D in the mechanism. On the other hand, the observation that the changes occurred in the absence of vitamin D as well as in its presence would indicate that dietary vitamin D was not a major factor in this process. In an initial study the effect of estrogen treatment on two different strains of mice fed commercial food mixtures was investigated.

Experimental. Male mice of two strains, CHI and C3H, were employed in the present investigation. The animals were bred from stock animals obtained from the colony maintained by Dr. Leonell Strong of Yale University. The groups of animals fed a vitamin D-deficient diet were protected from light by cardboard shields, and the cages were provided with wire screens to keep the animals off the droppings. Vitamin D-free diets of two different types were used. One of the diets with a normal Ca:P ratio and deficient in vitamin D was identical in composition with that employed by Sherman and Stiebeling[3] in some of their studies on vitamin D. The other deficient diet, U.S.P. XII Rachitogenic Diet No. 1, possessed the abnormally high Ca:P ratio which is characteristic of many rachitogenic diets. α-Estradiol dipropionate[†] was the estrogenic agent administered in this study and the duration of treatment was 12 weeks. At the end of this time the mice were killed with ether and autopsied. One femur of each animal was removed, dried, and ground as a gross specimen. The pubic bones were inspected.

The diets fed to the several groups of experimental animals and the amounts of estrogen received by the mice in the different groups are shown in Table I. The experimental results as shown in Table I mark an interesting strain difference between the CHI and the C3H mice. The former show no obliteration of the marrow cavity of the femur when they are treated with estrogen and fed either Purina Fox Chow or Pratt's Nurishmix, although some resorption of pubic bone was observed. On the other hand, mice of the latter strain showed complete obliteration of the marrow cavity when treated with estrogen and fed a stock diet. For this reason, mice of the C3H strain were used for the vitamin D study. The treatment of the mice and the

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[*] Ciba Fellow in Endocrinology.

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[2] Hisaw, F. L., *J. Exp. Zool.*, 1925, **42**, 411.

[3] Sherman, H. C., and Stiebeling, H. K., *J. Biol. Chem.*, 1929, **83**, 497.

[†] We would like to thank Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Incorporated, for the generous supply of α-estradiol dipropionate.

TABLE I.

Effect of α-Estradiol Dipropionate on Endosteal Bone Deposition in Different Strains of Mice and in Mice Fed Diets Deficient in Vitamin D.

Strain	No. of mice completing 12-week period	Diet	Amt of estrogen inj. subcut. once a wk,[1] γ	Obliteration of marrow cavity of femur after 12 wks,[2]	Resorption of pubic bones after 12 wks[3]
CHI	12	Purina Fox Chow[4]	50	0	++
CHI	9	’’ ’’ ’’ [4]	0	0	0
CHI	12	Pratt’s Nurishmix[4]	50	0	++
CHI	10	’’ ’’ [4]	0	0	0
C3H	10	Purina Fox Chow[4]	50	++++	++
C3H	11	’’ ’’ ’’ [4]	0	0	0
C3H	10	Pratt’s Nurishmix[4]	50	+++[5]	++
C3H	8	’’ ’’ [4]	0	0	0
C3H	13	Vitamin D-deficient diet[6]	50	++++[7]	+
C3H	12	’’ ’’ ’’ [6]	0	0	0
C3H	11	Vitamin D-deficient diet[6] + viosterol[8]	50	++++	+
C3H	12	’’ ’’ ’’ [6] + ’’ [8]	0	0	0
C3H	13	Vitamin D-deficient diet[9]	50	++++	++
C3H	12	’’ ’’ ’’ [9]	0	0	0

[1] In 0.05 cc sesame oil; control animals received the oil alone.

[2] 0 = none; +++ = slight persistence of proximal end of marrow cavity; ++++ = complete obliteration.

[3] 0 = none; + = 1 to 2 mm pubic ligament; ++ = 2.1 to 4 mm.

[4] Supplemented semi-weekly with lettuce.

[5] 2 of animals had ++++.

[6] Sherman Stiebling Diet.

[7] 3 of animals had +++.

[8] 50 units of viosterol in 0.05 cc corn oil injected subcutaneously weekly.

[9] U.S.P. XII Rachitogenic Diet No. 1.

results of this study are likewise shown in Table I. It is noteworthy that the mice treated with estrogen and fed a vitamin D-deficient diet exhibited the typical changes in the femur whether or not they were receiving viosterol.

Summary. Obliteration of the marrow cavity of the femur by endosteal bone and some resorption of pubic bone was observed in C3H mice fed commercial stock diets and treated with estrogen. Comparable treatment of CHI mice did not cause obliteration of the marrow cavity of the femur although some resorption of pubic bone was noted.

C3H mice treated with estrogen and fed a vitamin D-deficient diet supplemented with viosterol showed the typical changes in the femur. This result was also obtained in animals fed the same diet unsupplemented with vitamin D. Under the conditions of these experiments dietary vitamin D did not play a major role in the mechanism of endosteal bone deposition in the femurs of mice treated with estrogen.

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Effect of Cecectomy and Succinylsulfathiazole on Vitamin K Synthesis.*

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Great interest is centered on the synthesis of essential nutrients by microörganisms of the digestive tract.[1-6] The cecum, aptly referred to as a "fermentation vat,"[6] is generally regarded as the principal site of this important function in the rat.

Although quite a few reports have been made concerning the inhibitory effects of certain sulfonamides on the enteric synthesis of various vitamins very little experimental work has been done on the role of the cecum as a favorable site for such activity. The latter studies have been concerned only with the synthesis of B-complex vitamins[7-9] and the utilization of phytin phosphorus.[10]

During the past year our investigation of factors affecting vitamin K synthesis in the intestinal tract[11] has included observations on cecectomized rats. The timeliness of certain of our findings prompts this short communication.

Stock rats about 35 days old were started on a vitamin K-free diet of the following composition: casein (Smaco, vitamin-free) 18, glucose (Cerelose) 66, salt mixture 4, ether-extracted brewer's yeast 7, corn oil 3, cod liver oil 2, and choline chloride 0.04. After one week these animals were cecectomized and two days after the operation succinylsulfathiazole was added to the vitamin K-free diet at a level of 1% replacing an equal amount of glucose. Four weeks after cecectomy determination of the plasma prothrombin clotting time was started at biweekly intervals using a modification of Quick's method.[12] The blood was obtained by cardiac puncture under ether anesthesia.

Severe hypoprothrombinemia occurred in 14 out of 17 cecectomized animals fed the vitamin K-free diet containing succinylsulfathiazole. Within 3 months nearly all had died from hemorrhage due to severe hypoprothrombinemia. Seven out of 9 littermates cecectomized at the same time and fed the diet without succinylsulfathiazole had normal prothrombin clotting times. The remaining 2 showed only a slight increase in prothrombin time. Similarly, unoperated (not cecectomized) rats fed the vitamin K-free diet containing succinylsulfathiazole had only an occasional and very mild hypoprothrombinemia.

In cecectomized rats fed succinylsulfathiazole the elevated prothrombin clotting time consistently returned to normal upon the addition of 2-methyl-1,4-naphthoquinone to the

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The succinylsulfathiazole was generously supplied by Sharp and Dohme, Inc., through the courtesy of Doctors W. A. Feirer and J. Wm. Crosson.

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diet. This is additional evidence that vitamin K deficiency had been produced.

Over half a million pounds of sulfonamides were used in 1940,[13] and as a result of the proven usefulness of these drugs more may be used in the future. However, certain harmful effects cannot be ignored. In addition to other reported injurious consequences, we have noted a hemorrhagic tendency occurring under the influence of the sulfonamide derivatives which, because of low solubility and very slow absorption, remain in the lumen of the intestinal tract and act as efficient bacteriostatic agents. At the same time they interfere with the bacterial synthesis of vitamin K in the intestinal tract and, if administered for several weeks, may reduce the prothrombin content of the blood when the diet is extremely restricted.

At the beginning of our studies most of the animals died of hemorrhage about the third postoperative day. These rats had been fed the vitamin K-free diet containing succinylsulfathiazole for over a week prior to cecectomy. Their death was attributed to low plasma prothrombin resulting from K-avitaminosis. Such an outcome was subsequently avoided by performing the cecectomy before administration of the sulfonamide, or by administration of vitamin K.

These findings demonstrate that rats fed a vitamin K-free diet containing yeast suffer from severe and often fatal hypoprothrombinemia when succinylsulfathiazole is added and the volume of the lower bowel is greatly reduced by cecectomy. However, cecectomy in the absence of succinylsulfathiazole may lead to only a slight increase in prothrombin clotting time, if any, suggesting appreciable vitamin K synthesis in other parts of the digestive tract under normal conditions.

Gatch and his assistants[14] noted postoperative hemorrhage in a few patients given succinylsulfathiazole pre- and post-operatively and alleviated the condition by administration of synthetic vitamin K.

In the light of the above experimental and clinical observations surgeons should keep in mind the possibility that patients given succinylsulfathiazole or closely related compounds over long periods of time may develop hemorrhage as a result of the bacteriostatic effect which thus reduces vitamin K synthesis in the intestinal tract, and that the administration of vitamin K alleviates the hypoprothrombinemia and prevents the post-operative hemorrhage resulting therefrom. However, the amount of succinylsulfathiazole used and the length of the period of its administration pre- and post-operatively influence the occurrence of hypoprothrombinemia and the consequent hemorrhagic tendency. Large amounts used over a long period of time are more liable to cause hypoprothrombinemia, especially if the diet is so restricted as to contain insufficient amounts of vitamin K or none at all, but small amounts of the drug or a short duration of its administration is not as likely to cause hypoprothrombinemia.

The authors wish to express their appreciation to Mr. Robert Koons for his technical assistance.

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